



Department of Defense
Breast Cancer Research
Program Meeting

June 25–28, 2008
Baltimore Convention Center
Baltimore, Maryland

Time for Action

PROCEEDINGS

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Era of Hope

Department of Defense
Breast Cancer Research Program Meeting

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June 25–28, 2008
Baltimore Convention Center
Baltimore, Maryland



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

MAY 05 2008

Office of the Commander

Dear Colleagues:

Welcome to the U.S. Department of Defense (DOD) Breast Cancer Research Program (BCRP) 2008 Era of Hope meeting. This meeting represents the collaborative efforts of many people to reduce breast cancer's impact on the lives of all who are affected by the disease. The 2008 meeting highlights the multidisciplinary, high-impact, innovative research that the DOD BCRP has funded in our efforts to eradicate breast cancer.

One of the DOD BCRP's most notable hallmarks is the unique collaboration among the research community, breast cancer consumers, and the U.S. Government. This collaborative spirit represents the culmination of hard work initiated and sustained by survivors and advocates, Congress's decision to continue research funding for breast cancer, and the dedication of scientists and clinicians to the program. This Era of Hope meeting demonstrates the U.S. Army Medical Research and Materiel Command's commitment to manage the DOD BCRP in a manner responsive to the vision and equal to the dedication of all of our partners.

To facilitate communication and encourage greater interaction among the many diverse disciplines in attendance, the Era of Hope 2008 meeting focuses on "Time for Action," and is organized around three unifying themes: Risk and Prevention Across the Spectrum of Breast Cancer, Breast Cancer Diagnosis – What's on the Horizon, and Managing Breast Cancer Across the Spectrum of Disease. All BCRP award recipients from fiscal years 1992-2006 were invited to this meeting to present their research findings and discuss promising directions in breast cancer research. Breast cancer consumers, who have been an integral part of the DOD BCRP since its inception, are serving side-by-side with the scientists as co-chairs and speakers in all sessions at this meeting.

My staff and I thank you for your continuing partnership and dedication in our efforts to eliminate this disease, sustain health, and improve the quality of life for those living with breast cancer.

Sincerely,

A handwritten signature in black ink, appearing to read "George W. Weighman", is positioned above the printed name.

George W. Weighman
Major General, Medical Corps
Commanding General



REPLY TO
ATTENTION OF

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
1077 PATCHEL STREET
FORT DETRICK, MD 21702-5024

June 25, 2008

Congressionally Directed Medical Research Programs

Dear Colleagues:

It is with great pleasure and honor that the Department of Defense (DOD) Breast Cancer Research Program (BCRP) highlights the research it has funded at this fifth Era of Hope meeting – Time for Action. The Era of Hope 2008 Meeting marks the 16th anniversary of the DOD BCRP. During this time, the DOD BCRP has emerged as a leader in funding innovative, high-impact, multidisciplinary research focused on breast cancer. The scientific excellence and innovation of the research presented at this Era of Hope meeting are testimony to the many talented and creative researchers supported by the DOD BCRP.

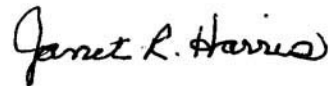
The DOD BCRP was created in response to the concerns of those directly affected by breast cancer. Breast cancer survivors and advocates have had a tremendous impact on this program with their participation in all aspects of the vision setting and review processes. The fruitful collaboration among survivors, advocates, scientists, clinicians, and the federal government is one of the DOD BCRP's most significant achievements. I believe that the success of this partnership is due to their shared dedication and commitment to eradicate breast cancer.

I express my deepest gratitude to all of the people who have participated in the DOD BCRP, to all who embrace this time as a "Time for Action":

- Breast cancer survivors and advocates, whose courage and commitment created this program. They instill their passion, inspiration, and vision of eradicating breast cancer into the program.
- Researchers funded by the program who, through their research, are rising to the challenge of eliminating breast cancer. They provide hope for one day, finding a cure.
- Members of the Integration Panel, present and past, who craft a responsive, dynamic, and comprehensive program every year through fiscally responsible investment strategies and award mechanisms supporting these strategies and identify the research to most effectively move us closer to a cure.
- Members of the DOD BCRP peer review panels, who have met the challenge of reviewing over 35,000 proposals during the past 16 years. Their expertise and perseverance have assisted us in finding the best research to move us closer toward the program's vision.
- Members of the DOD, the U.S. Army Medical Research and Materiel Command, the BCRP Program Management Team, and support staff, whose energy, enthusiasm, and diligence sustain the DOD BCRP.

I gratefully acknowledge the intellectual and visionary contributions of all of these individuals who are committed to the DOD BCRP as it blazes new trails toward eradicating breast cancer. This fifth Era of Hope meeting is the culmination of 16 years of progress by the DOD BCRP in the fight against breast cancer. I thank you for your participation in this "Time for Action."

Sincerely,

A handwritten signature in black ink that reads "Janet R. Harris". The signature is written in a cursive, flowing style.

Janet R. Harris, Ph.D., RN
Colonel, U.S. Army Nurse Corps
Director

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INNOVATOR, EOH SCHOLAR, EOH POSTDOC

Poster Session P1

P1-1: MECHANISMS OF BREAST CANCER RECURRENCE

James V. Alvarez
University of Pennsylvania

The natural history of breast cancer is frequently characterized by recurrence of the primary tumor following initial therapy. Recurrent tumors, which can arise locally or at distant sites, are responsible for the majority of deaths from breast cancer. At present, very little is known about the cellular processes or molecular pathways that are involved in tumor recurrence. Consequently, a detailed understanding of the mechanisms underlying recurrence would contribute significantly to the treatment of breast cancer.

To better understand the mechanisms of tumor recurrence, our laboratory has generated mouse models that faithfully recapitulate many key aspects of breast cancer recurrence in humans. These models employ the tetracycline-inducible system to allow for mammary-gland specific, doxycycline-inducible expression of various oncogenes, including Myc, Neu, and Wnt1. Oncogene expression consistently leads to formation of mammary gland tumors, and de-induction of the oncogene frequently leads to complete regression of the primary tumor. However, following a period of latency during which the tumors remain nonpalpable, tumors frequently recur, and these recurrent tumors are no longer dependent upon the oncogene that initiated their growth. These models thus provide a tractable system in which to investigate the cellular and molecular mechanisms of breast cancer recurrence.

To gain insight into these mechanisms, we first performed gene expression profiling to compare primary tumors initiated by Myc, Neu, and Wnt1 with their corresponding recurrent tumors. We identified a cohort of genes whose expression was significantly altered in recurrent tumors in all 3 models. Principal component analysis revealed that recurrent tumors arising in all 3 models had very similar transcriptional profiles and that these profiles differed substantially from the corresponding primary tumor. This suggests that the recurrent tumors arising in these models may employ similar pathways.

We next wished to determine whether these genes are also involved in recurrence in humans. To do this, we analyzed the expression of these genes in several published human breast cancer datasets for which survival data were available. Using Kaplan-Meier analysis, we found that a substantial number of these genes correlated with survival in human patients. These genes will be tested in an orthotopic tumor recurrence model, allowing for the functional validation of these candidates.

In conclusion, the system described has allowed for the identification of candidate genes likely to play a role in breast cancer recurrence. These genes may identify common pathways by which diverse primary tumors recur and are clinically relevant as they are associated with survival in human breast cancer patients. Given the critical importance of better understanding the process of tumor recurrence, our studies have the potential to identify novel therapeutic targets that may improve the treatment of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0420.

P1-2: THE USE OF POLY(ADP-RIBOSE) POLYMERASE (PARP1) INHIBITORS IN THE TREATMENT OF BREAST CANCER

Joseph A. De Soto
Uniformed Services University of the Health Sciences

Introduction: In the United States, breast cancer is the most prevalent cancer in women and the number two cause of cancer death. Most of our current chemotherapeutic approaches tend to be based on either inducing DNA damage or inhibiting the synthesis of DNA. These approaches are both highly toxic and have relatively poor efficacies. Recently, it was shown that BRCA-associated tumors might be highly susceptible to blockers of DNA repair called poly(ADP-ribose) polymerase (PARP) inhibitors. Here we evaluate the efficacy of PARP-1 inhibitors alone and in combination with DNA disruptors to inhibit human breast cancer.

Methods: In this study, BRCA1 +/+, +/-, and -/- breast cancer cell lines were exposed for 72 hours to the PARP1 inhibitor AG14361 and the PARP1 and 2 inhibitor ABT888 alone and in combination with anti-metabolites, alkylating agents, topoisomerase inhibitors, antimetabolic agents, platinum-type alkylating agents, and natural products. The IC₅₀ values were then calculated. Human breast cancer cells were then implanted into athymic nude mice and treated with PARP inhibitors alone or in combination with the promising standard chemotherapeutic agents as determined from our in vitro studies.

Results: Both ABT-888 and AG14361 inhibited the growth of both BRCA1-associated and wild-type breast cancer in a relatively nonspecific manner. The PARP-1 and 2 inhibitor ABT-888 antagonized the cytotoxic effects of the taxanes but was additive or synergistic with the topoisomerase inhibitors, 5-FU, and the platinum-type alkylating agents. The PARP1 inhibitor AG14361 was additive or synergistic with 5-FU, taxanes, topoisomerase inhibitors, and the platinum-type alkylating agents. In xenograft human/mouse studies, complete remission of breast cancer was observed com-

binning with the PARP1 inhibitor AG14361 with selected chemotherapeutic agents. In addition, upon necropsy, there was less observed toxicity in noncancerous tissue.

Conclusions: PARP-1 inhibitors when combined with some standard chemotherapeutic agents may be useful in the treatment of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0388; National Institutes of Health; and Uniformed Services University of the Health Sciences.

P1-3: SOMATIC MUTATION AND AMPLIFICATION OF NOTCH3 IN HUMAN BREAST CARCINOMA

Sofia Gruvberger-Saal, Lao H. Saal, Hanina Hibshoosh, and Ramon Parsons
Columbia University College of Physicians and Surgeons

The catalogue of driver mutations, amplifications, and deletions in breast carcinoma (BC) is incomplete, and the relationship of specific lesions to BC subtypes is poorly characterized. We have recently found loss of PTEN to be common in basal-like BC and to be grossly mutated in BRCA1-associated basal-like tumors. In a search for other potential driver genes in human BC biopsies utilizing DNA copy number analysis (aCGH and SNP arrays) and direct sequencing, we have now identified frequent mutations and/or amplification of *NOTCH3*, a member of the Notch receptor family, which are known to be involved in cell proliferation, cell differentiation, and cell-fate decisions. Currently, we are conducting shRNA knock-down experiments of *NOTCH3* in BC cell lines with high endogenous expression and are studying the effects on cell proliferation and colony formation. The activation properties of the somatic mutations are being investigated in a dual-luciferase assay using a NOTCH-responsive reporter, and their transforming abilities are being ascertained in human immortalized mammary MCF-10A cells in culture. In addition, we are measuring *NOTCH3* mRNA expression in primary breast tumor samples by cDNA microarray profiling and investigating its relationship to patient outcome. Our preliminary results suggest that *NOTCH3* is important in the pathogenesis of a subset of BCs and that high expression is associated with a significantly worse prognosis. These results may be clinically significant given the availability and current development of agents that target the NOTCH pathway, for example, γ -secretase inhibitors. Identifying genes and pathways contributing to the initiation and progression of carcinoma in the different subtypes of breast cancer will give new important information for guiding targeted therapy and treatment decisions.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0401 and National Institutes of Health (5T32 GM07367-29, CA082783, and CA097403).

P1-4: USE OF OPTICAL SPECTROSCOPY TO MONITOR DELIVERY OF A NOVEL CELL CYCLE INHIBITOR

Gregory M. Palmer
Duke University

Background: This study investigated the use of optical spectroscopy to monitor the physiologic response and pharmacokinetics of MKC-1, a novel cell cycle inhibitor. MKC-1 is fluorescent, which presents an opportunity to dynamically monitor the pharmacokinetics of this agent.

Methodology: 4T1 murine mammary carcinoma cells were grown in the flank of nude mice. After tumor establishment, MKC-1 was administered daily via gavage. Diffuse reflectance and fluorescence spectra were acquired transdermally via a fiber optic probe over UV-visible. A Monte Carlo modeling algorithm was employed to account for the effects of absorption and scattering, and the MKC-1 fluorescence was spectrally resolved from the autofluorescence.

Results: Figure 1 shows the intrinsic fluorescence spectra of tissue over time for a single animal upon administration of MKC-1 (at t=0). After treatment with MKC-1, a distinct fluorescence signature appears at 545 nm emission, which peaks at around 1–2 hours after treatment and then slowly decays. Figure 2 shows one time course of the MKC-1 fluorescence for the group of eight animals with treatment given at t=0.

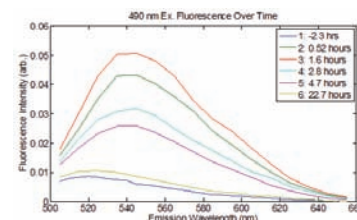


Figure 1. Fluorescence emission spectra acquired as a function of time after MKC-1 treatment.

The extracted fluorescence intensity can be seen to peak between ~2–7 hours and varies widely both in peak magnitude and time delay.

Conclusions: We have demonstrated the ability of optical spectroscopy to elucidate the kinetics of drug delivery noninvasively. Future work will involve determination of what MKC-1 is bound to when exhibiting this fluorescence, calibration to absolute concentration values, as well as analysis of the effects of MKC-1 on the physiologic parameters reported by optical spectroscopy, particularly tumor oxygenation. This work could potentially be used to improve drug delivery and enable personalized therapy based on an individual's unique pharmacokinetic profile.

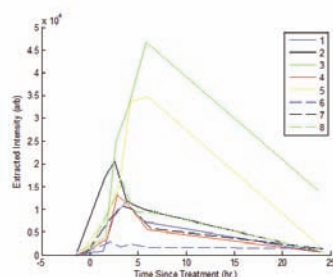


Figure 2. MKC-1 fluorescence intensity (concentration) profile as a function of time for 8 mice.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0355.

PI-5: CHARACTERIZATION OF Mcs5A, A NON-CODING BREAST CANCER SUSCEPTIBILITY LOCUS

Bart M. G. Smits,¹ Jill D. Haag,¹ David J. Samuelson,² and Michael N. Gould¹
¹University of Wisconsin, Madison and ²University of Louisville

The heritable fraction of breast cancer susceptibility includes multiple low-penetrance high-population frequency modifier alleles that are largely unknown. In a comparative genomic approach to identify novel susceptibility loci, we employed QTL mapping in a rat mammary carcinogenesis model and subsequent fine-mapping using congenic rat lines. We identified an entirely non-coding mammary carcinogenesis susceptibility locus, Mcs5a. Mcs5a is a compound QTL consisting of minimally two genetic elements (Mcs5a1 and 5a2) that must be on the same chromosome to affect susceptibility. Mcs5a1 (~32 Kb) includes 5' UTR and intronic sequences of the F-box containing gene, Fbxo10. Mcs5a2 (~84 Kb) contains primarily intergenic sequence and 5' UTR of the FERM/PDZ-domain containing gene, Frmpd1. We conducted a large case-control association study (n=12,000) and found common risk-associated alleles in both human orthologous loci. In the rat, none of the genes within 1 Mb surrounding Mcs5a is differentially expressed in the mammary gland, suggesting a mammary cell nonautonomous gene regulatory function of Mcs5a. Indeed, Fbxo10 showed a differential expression pattern in the thymus that is consistent with the susceptibility phenotype. Data from flow cytometry experiments and gene expression arrays strongly suggest involvement of CD3+ T cells. Hence, we hypothesize that the genetic elements in Mcs5a1 and Mcs5a2 physically loop together to regulate Fbxo10 expression in CD3+ T cells. To demonstrate the physical interaction, we adopted the chromatin conformation capture (3C) assay and applied it to a human leukemic T cell line (JURKAT) and rat CD3+ T cells. In the human T cell line, the MCS5A1 element containing the risk-associated marker and correlated polymorphisms loops over to the risk-associated and correlated SNP region in MCS5A2. This chromatin folding over ~50 kb likely brings together the regulatory elements, which currently undergo functional validation. The chromatin folding structure in rat T cells is more complex, suggesting involvement of multiple regulatory elements. Together with a series of chromatin immunoprecipitations on microarrays, this approach will aid in the discovery and initial annotation of most of the regulatory elements in Mcs5a. Functional studies with all alleles involved will likely reveal the causative polymorphisms of the differential regulation of Fbxo10 in T cells. To begin to unravel the genetic complexity underlying Mcs5a and breast cancer susceptibility in general, it will be important to determine the function of the non-coding alleles in our animal model and evaluate them in human cell systems.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0404 and National Institutes of Health.

PI-6: IN VIVO ANALYSIS OF ALTERNATIVE MODES OF BREAST CANCER CELL INVASION

Donald White,¹ William J. Muller,² Alan Ashworth,³ and Christopher J. Marshall¹

¹Institute of Cancer Research, London, ²McGill University, and ³Breakthrough Breast Cancer Institute, London

The dissemination of breast cancer cells from a primary lesion to distant sites such as brain or bone presents the most serious threat to patient survival. As part of the metastatic process, cells must migrate from the primary tumor and invade into adjacent tissue and vasculature. The way by which tumor cells navigate through the fibrous extracellular matrix (ECM), however, is not clear. One proposed mechanism involves the loss of epithelial properties and the acquisition of a more motile, fibroblast (or mesenchymal)-like cell. Mesenchymal cells are characterized by an elongated, rather than cuboidal, morphology, as well as prominent actin-rich protrusions at the leading edge of the cell. Adhesive forces at the front of the cell, together with

coordinated waves of actomyosin contraction and retraction of the trailing edge, drive forward migration of these cells. In addition, the foremost protrusions are often rich in protease activity which facilitates invasion by degrading the ECM at the front of the cell. Until recently, this epithelial-mesenchymal transition (EMT) was generally regarded as the predominant mechanism of tumor cell migration and invasion. Recent reports, however, describe an alternative form of movement in which the cells acquire a rounded or amoeboid morphology, allowing them to squeeze through interstitial spaces of the ECM. This form of movement is of clinical interest since it circumvents the need for pericellular proteolysis and may therefore complicate our ability to block metastasis using protease inhibitors. Importantly, it has been shown that various tumor cell lines have the capacity to switch from the elongated to the amoeboid form of movement in the presence of protease inhibitors. This mesenchymal-amoeboid transition (MAT) involves downregulation of Rac1 activity, which would normally drive formation of membrane protrusions through the assembly of a branched actin network at the leading edge of the cell. In turn, activation of the Rho kinases ROCK1 and ROCK2 leads to high levels of actomyosin contractility, driving formation of the highly contractile, rounded morphology. Although this phenomenon has been demonstrated in cell culture, it is not known if the MAT can occur in vivo. The purpose of our proposed project, therefore, is to determine the types of movement employed by tumor cells in 3 well-established mouse models of human breast cancer. Mammary tumors in these models are driven by expression of the activated erbB-2/neu or PyVnT oncogenes or by loss of the BRCA1 tumor suppressor. Analysis of explanted cells from these tumors reveals the capacity to use either elongated or rounded forms of movement ex vivo, depending on the substratum. The use of pharmacological inhibitors confirms that these forms of movement require Rac1 and Rho/ROCK activity, respectively. Using a lentivirus-shRNA approach, we are currently targeting these pathways in vivo to determine the impact on local invasion and metastasis and to determine whether one pathway can compensate for cell movement following loss of the other pathway in vivo. We hope that this work will lead to a more detailed understanding of how modes of tumor cell movement in vivo contribute to breast cancer progression thereby identifying more effective prognostic markers and therapeutic targets for this devastating disease.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0349.

PI-7: RECENT ADVANCES IN DIGITAL TOMOSYNTHESIS MAMMOGRAPHY RECONSTRUCTION

Yiheng Zhang, Heang-Ping Chan, Berkman Sahiner, Jun Wei, Yi-Ta Wu, Jun Ge, Chuan Zhou, and Lubomir Hadjiiski
 University of Michigan

Background/Purpose: Digital tomosynthesis mammography (DTM) is one of the most promising techniques that can potentially improve early detection of breast cancers. It can provide quasi three-dimensional (3D) volumetric information, reduce the camouflaging effects of overlapping dense tissue, and improve lesion conspicuity. We are developing advanced and efficient DTM reconstruction algorithms for optimizing image quality and minimizing image artifacts. Our recent works include: (1) quantitative comparison of different algorithms in breast tomosynthesis reconstruction, (2) utilization of breast shape information to achieve highly efficient iterative computation, (3) artifact reduction methods to remove image artifacts from multiple sources, and (4) investigation of the impact of DTM imaging parameters on depth resolution.

Method: DTM acquires a sequence of low-dose projection-view digital mammograms over a limited angular range. DTM reconstruction of the breast volume is a limit-angle cone-beam tomographic problem, providing thin slices of the breast parallel to the detector plane. Several representative 3D reconstruction algorithms are investigated, including back-projection method, algebraic reconstruction techniques, and statistical reconstruction method. A large variety of image quality measures, such as contrast-to-noise ratio, artifact spread function, and depth resolution are developed and used in quantitative evaluation. For iterative reconstruction techniques, computational effort is an important issue. Two-dimensional breast boundaries on all projection-view (PV) images are detected automatically and used to generate the 3D breast shape. The information is used to restrict the iterative reconstruction to be performed only within the breast volume to avoid unnecessary computation. Due to the limited angular range and the truncated projection images especially at large angles, reconstructed DTM slices contain strong truncation artifacts that obscure the textures and adversely affect both radiologists' reading and computerized processing. We have developed methods to suppress the truncation artifacts caused by the changing imaged region of each PV and the glaring artifacts caused by the truncated imaged volume modeled in the reconstruction. Finally, we investigate the impact of DTM imaging parameters on the depth resolution that determines the degree of separation between image features in different depths of the breast. In this study, a GE prototype DTM system was used to acquire 21 PVs in 3° increments over a ±30° angular range for all breast phantoms and patient cases.

Results: A simultaneous algebraic reconstruction technique (SART) can achieve high image quality for DTM reconstruction. The computational effort of SART can be substantially reduced by using breast shape information. Both the boundary artifacts and truncation artifacts can be effectively reduced, and the overall image quality is

significantly improved. Among different imaging parameters, the depth resolution is found to be strongly affected by the tomosynthesis angular range.

Conclusion: Our advanced techniques can provide high image quality of DTM reconstruction and achieve significant improvement in computational efficiency and reducing image artifacts, which is expected to further improve breast cancer detection by this new imaging modality.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0324.

PI-8: OBSERVING THE LONGITUDINAL EFFECTS OF DOXORUBICIN ON MOUSE TUMOR PHYSIOLOGY VIA DIFFUSE OPTICAL SPECTROSCOPY

Karthik Vishwanath and Nirmala Ramanujam
Duke University

Doxorubicin is an actively used neoadjuvant chemotherapeutic agent in the treatment and care of breast cancer since these regimens have shown higher response rates in both progression-free and overall survival in women with advanced breast cancer. Treatment efficacy can be significantly improved if the oncologist can determine early-response in the patients. Here, we study if diffuse optical spectroscopy (DOS) can be used as a means to gauge tumor response in an animal model of breast cancer.

Fiber-based DOS was used to measure the diffuse reflectance between 350–600 nm from 4T1 murine breast carcinoma tumors that were implanted in the flank of 50 nude mice. The animals were evenly randomized into control and treatment groups (with each group containing 25 animals). The treated group received a maximum tolerated dose (MTD) dose (10 mg/kg, i.v.) of doxorubicin while the control group received an equivalent volume of saline. The tumors in both groups were then measured using the fiber-optic spectrometer on days 0, 2, 5, 7, 10, and 13. Day 0 represented the baseline measurement and was made just before the animals received treatment. During the course of optical measurements, the animals were anesthetized and immobilized via isoflurane breathing (1.5% isoflurane gas mixed with oxygen). The measured diffuse reflectance was quantified using a previously developed inverse Monte Carlo algorithm to obtain the tissue absorption and scattering coefficients. The extracted absorption coefficients yield concentrations of oxygenated hemoglobin (HbO₂) and deoxy-hemoglobin (HHb). From these we can calculate the total hemoglobin concentration (THb = HHb + HbO₂) and the hemoglobin saturation (SO₂ = 100*HbO₂/THb) for both groups, across time. It was seen that relative to day 0 baseline values, treatment with doxorubicin significantly increased overall SO₂ in tumors on each of the days 2, 7, 10, and 13. However, the THb content was higher in the control group on days 2 and 5 relative to the treated group, but decreased on day 13. These data indicate that the MTD dosage of doxorubicin impacts the development of tumor vasculature in the treated animals.

Five randomly chosen animals from each group were removed for immunohistochemical (IHC) analysis on days 0, 5, 10, and 13. The tumors from these animals were excised and snap-frozen. These tumors were subsequently sectioned in a cryomicrotome (in 5 µm slices) and imaged to yield the necrotic fraction (from H&E staining) and hypoxic fraction (from pimonidazole staining). The hypoxic fraction was obtained by dividing the positively stained area by the overall viable tissue area. The obtained hypoxic fraction from IHC was positively correlated ($r^2 = 0.51$, p -value < 0.05) with HHb concentration measured optically. The necrotic fraction from IHC was positively correlated with the scattering coefficient ($r^2 = 0.67$, p -value < 0.01).

A previous study that monitored the hemoglobin content and blood oxygenation in breast cancer patients undergoing neoadjuvant chemotherapy with doxorubicin using DOS, determined that patients that responded to the therapy showed an overall drop of ~27% in the Hb concentrations 1 week after the start of treatment. Here, we found similar trends in the Hb concentration, which fell by ~29%, 7 days post treatment in the treated group. Future work will study if the optical measurements are able spot early-responders.

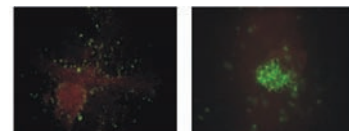
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0363.

PI-9: CANCER-STROMAL CELL INTERACTIONS

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Cancer-stromal cell interactions play an important role in the growth and invasion of tumors. Mature adipocytes are abundant in breast tissue; it has been suggested that they affect the biological behavior of some carcinoma cell lines through the production of cytokines such as leptin, adiponectin, tumor necrosis factor- α , adipsin, insulin-like growth factor-II, and collagen type VI. In vivo, mature adipocytes produce estrogen and aromatase, which also affect the growth of breast carcinoma cells through paracrine signaling.

We previously reported a co-culture method, developed to regulate the in vitro adipogenesis of mouse multipotent stromal cells (D1s). D1s were maintained in the presence of the bovine mammary epithelial cell (MAC-T) conditioned medium and adipogenic cocktail. After treatment with MAC-T conditioned medium and adipogenic cocktail for 14 days, no peri-nuclear lipid arrangements or ap2 secretions were detected. We thus hypothesized that the interaction of D1s and estrogen-dependent breast cancer cells (MCF-7s) might be altered simply by regulating the adipogenesis of D1s in co-culture with MCF-7s. Since D1s may be regulated to differentiate into adipocytes (in the presence of adipogenic cocktails) or pre-adipocytes (in the presence of MAC-T conditioned medium), the in vivo behavior of MCF-7s would likely also be affected by these conditions. Thus, co-cultures of D1s and MCF-7s were maintained in standard culture medium, adipocyte differentiation medium, and MAC-T conditioned medium for 12 days, and then were treated with tamoxifen for 3 days. A Vybrant Apoptosis Assay was used to pinpoint the apoptotic cells. The results showed that MCF-7s cultured with adipocytes (Figure a) were less susceptible to tamoxifen than pre-adipocytes (Figure b). This co-culture method has the potential to create a microenvironment that enables cytokine and extracellular matrix molecule production by emulating in vitro the heterotypic cell interactions found in the breast cancer process.



Green fluorescence denotes apoptotic cells (320X magnification)

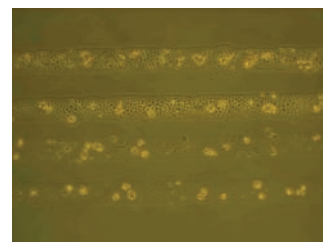
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0379.

PI-10: THE MODIFIED INKJET CELL PRINTER AS A TOOL FOR 3-DIMENSIONAL BREAST TISSUE MODELING

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Clemson University

The current “gold standard” for tumor modeling involves the suspension of cells within gel-like matrices; though these systems appear to be superior to traditional 2-dimensional models, they lack the rigidity necessary to allow for normal functioning of anchorage-dependent breast cells. Tissue engineering strategies may be employed in the development of in vitro breast tissue models that have potential for use in testing of drug therapies and vaccines. Conventional cell seeding methods are inadequate in the development of tissue models because they involve the random placement of cells and therefore lack the precision necessary for spatial control. We have selected the modified inkjet printer as a tool for creating 3-dimensional in vitro models because it offers an inexpensive and high-throughput solution to microfabrication and because the printer can be easily manipulated to produce varying tissue attributes.

To demonstrate the resolution capabilities of inkjet cell printing technology, D1 (murine mesenchymal stem) cells were suspended in serum-free culture medium at a concentration of 5×10^6 cells/mL and printed in a straight line pattern onto glass slides. This pattern was retained on slides when lines were separated by a distance of 200 µm and pixel spacing was set at 50 µm. Figure 1 (100x total magnification) shows the potential for accurate and precise spatial control of cells, extracellular matrix components, soluble factors, and drugs, among other 3-dimensional culture additives. The figure shows D1 cells printed in a straight line pattern using 50 µm pixel spacing and 200 µm line spacing.



High resolution spatial control can be achieved using inkjet printing technology

The effect of printing on the permeability of D1 and MCF-7 (human breast cancer) cells was evaluated using a modified HP 540 series inkjet printer. Printed D1 and MCF-7 cells were collected in a 1 mL test tube immediately following printing, and 20 µL of the cell suspension was aliquoted into a separate tube at 0, 5, 15, 30, and 60 minutes following the print process. The aliquot was mixed with Trypan Blue dye, pipetted into a hemacytometer, and cells were counted and categorized based on whether they excluded the dye. Results indicated that cells became less permeable to the dye over the 60-minute period. At the 15-minute time point, approximately 50% of D1 cells and 55% of MCF-7 cells excluded the dye while 70% of D1 cells and 75% of MCF-7 cells excluded the dye at the 60-minute time point. Further investigation will promote understanding of cell permeability as a controllable parameter using inkjet printing technology in the laboratory.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0379.

P1-11: DISCOVERY OF POTENT INHIBITORS FOR BREAST CANCER MULTIDRUG RESISTANCE

Geoffrey Chang

Scripps Research Institute

Breast cancer is often considered to be one of the more chemo-responsive tumors. Many structural diverse cytotoxic drugs, when administered either as single agents or in combination, can induce remission in previously untreated breast cancer patients. While the overall response rate can be high, the duration of the response is relatively short (~10–15 months). Nearly all initially responsive breast tumors will eventually acquire a multidrug resistant (MDR) phenotype over time. We focus on one of the most dominant causes of breast cancer drug resistance: MDR efflux pumps that are imbedded in the cell membrane. Although the precise nature of this form of drug resistance remains unclear, the development of MDR in metastatic breast cancer is a significant cause of failure for several treatment regimens. Most cancer MDR reversing agents, however, have been lacking in their potencies and specificities.

In the last 2 years, we have discovered and developed a series of new inhibitors of P-glycoprotein (Pgp or human MDR1) that will specifically block drug efflux in close collaboration with the laboratories of Drs. Ina Urbatsch, Qinghai Zhang, and M.G. Finn. We have established the relative potencies of existing anti-cancer drugs and developed new inhibitors to reverse the problem of breast cancer MDR by Pgp. We envision that patients having MDR breast cancer tumors will take one or a “cocktail” of these potent MDR inhibitors along with well-established cancer drugs. When taken synergistically with existing cancer drugs, these inhibitors should prevent MDR allowing our current chemotherapies to achieve or even surpass their original potencies.

Our research combines x-ray crystallography, function, and chemistry for the design of new MDR inhibitors for breast cancer chemotherapy. We synthesize and test our compounds using in vitro functional assays and determine the co-crystal structures of Pgp with these inhibitors. The most important and significant result of the last year was the x-ray structure determination of Pgp to 3.8 angstrom in resolution. These structures provided an incredible foundation for the study of drug binding and the rational design of new inhibitors. We believe that this structural information will be very valuable for the future design of breast cancer MDR reversal agents. Our lab has also detailed the functional activity of these compounds using transport and ATPase assays to ascertain the potency of inhibition and for the testing of these potential inhibitors human MDR cancer cell lines.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0316 and National Institutes of Health (GM 61905).

P1-12: DYSYNCHRONOUS EXPRESSION OF STEM CELL MARKERS (CD90, CD133) AND CYTOKERATIN MARK BREAST CANCER STEM CELLS

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Background and Objectives: We have previously characterized a subset of highly tumorigenic breast cancer (BrCA) stem cells expressing CD90, CD44 and having resting morphology. We have also found cells of this phenotype among the nonhematopoietic (NH) fraction of normal bone marrow. Distinguishing cancer and normal stem cells is important from the standpoint of targeted therapy.

Methodologies: Freshly isolated clinical material (9 breast cancer malignant effusions (PE), 10 normal bone marrows (BM), 3 BrCA BM, 3 normal and 3 BrCA mobilized peripheral blood progenitor cell products) were separated on a density gradient, counted and stained for flow cytometric analysis of stem and differentiation markers (nucleated cells: DAPI; Hematopoietic: CD45-APC.Cy7, CD14+CD33+glycophorin-PE.Cy5; Epithelial: HEA-APC, intracellular pancytokeratin (CK)-FITC; Stem/Progenitor: CD90-PE.TxRed, CD117-PE.Cy7, CD133-PE). Two to ten million events were acquired on a CyAn cytometer. Spectral compensation and analysis were performed offline using a prototype version of VenturiOne software. Analytical results were exported to SYSTAT software and ANOVA was performed to compare prevalence of populations of interest between tissue sources.

Results to Date: We focused analysis on NH CD90+ cells, which were found at approximately the same frequency (0.13% of NH cells) in cancer and normal tissues. When divided into CK positive and negative fractions CD90+ CK+ cells were unique (p=0.001) to PE (0.33% of NH) and BrCA BM (0.07% of NH). CD133 expression on NH cells was also confined (p<0.0005) to PE (0.7%) and BrCA BM (0.5%), and in PE expressed bright CK. NH CD90 and CD133 populations had little overlap. CD117 expression was highest among NH BM cells (BrCA and normal). The great majority of CD117+ NH cells were CK negative and did not appear to mark a tumor population.

Conclusions: We found that CD90-positive breast cancer stem cells are readily distinguished from their normal counterparts by the dysynchronous expression of the epithelial differentiation antigen CK. Additionally, CK in conjunction with CD133

appears to mark a population of BrCA cells. Resting BrCA stem cells are hypothesized to comprise the therapy-resistant tumor compartment. As such, phenotypic profiles that distinguish these tumor stem cells from normal tissue stem cells may be useful both for differential targeting and for evaluation of clinical responses.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0182; Hillman Foundation; and Glimmer of Hope Foundation.

P1-13: COLLAGEN CROSS-LINKING INCREASES MATRIX STIFFNESS TO DRIVE BREAST TRANSFORMATION AND MODULATE TREATMENT EFFICACY

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Background/Objectives: Breast malignancy is associated with a desmoplastic response although the relevance of this phenotype to tumor behavior has been controversial. We and others showed that breast desmoplasia occurs prior to and can actively promote breast tumor progression and that tempering the desmoplastic response can inhibit breast cancer progression. Work supported by the DOD has enabled us to study the role of the extracellular matrix (ECM) and its integrin receptors in the pathogenesis and treatment efficacy of breast cancer. We have been exploring the effect of collagen density, organization, and posttranslation modifications, which stiffen the ECM in malignant transformation and treatment responsiveness of the breast.

Approach and Observations: We have applied natural and synthetic three-dimensional (3D) hydrogels with calibrated stiffness, as well as the MMTV-Her2/neu transgenic mouse model of breast tumorigenesis, nude mouse cleared fat pad and xenograft manipulations, shear rheology, non confined compression analysis and AFM measurements, traction force microscopy, and molecular and pharmacological manipulations to explore the role of matrix stiffness in breast tumor behavior. Our studies indicate that malignant transformation is preceded by a progressive increase in matrix stiffness mediated by collagen deposition, linearization, and lox-dependent cross linking, which collectively alter mammary epithelial cell (MEC) behavior by inducing cell-generated contractility to promote the assembly of focal adhesions. Altering integrin function using an auto-clustering mutant or manipulating cell-generated force through Rho, ERK, ROCK, and myosin showed that increasing myosin contractility and focal adhesions permits the oncogenes ErbB2 and Ras to drive MMP-dependent tumor cell invasion and modify radiation, chemo, and immune-dependent treatment responsiveness of breast tissues by enhancing integrin and growth factor-dependent signaling through ERK, PI3 kinase, and JNK. We found that increasing collagen cross-linking either in vivo or in 3D cultures induces the linearization of collagen fibrils, stiffens the ECM, and promotes the malignant transformation of premalignant oncogene expressing MECs. Inhibiting lysyl oxidase-dependent collagen cross-linking either pharmacologically or using function blocking antibodies reduced the ECM stiffness and decreased the incidence and prevented the progression of oncogenically driven breast tumors in vivo and in vitro in association with lower mechano-signaling in the MECs. Furthermore, matrix stiffness modulates treatment efficacy of breast tumors by enhancing radiation, immune, and chemotherapy apoptotic responsiveness through elevated integrin-dependent JNK signaling.

Conclusion: Our results indicate that collagen stiffening can contribute to breast tumor-associated desmoplasia and that ECM stiffness can promote breast tumor progression and modulate treatment responsiveness. These data suggest that protocols that yield information about ECM cross-linking and stiffness might prove useful for predicting which breast tumors might progress rapidly and how they might respond to treatment. Agents directed at inhibiting ECM stiffening might constitute an attractive adjuvant therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0330.

P1-14: THE GENETIC ARCHITECTURE OF BREAST CANCER SUSCEPTIBILITY AND ITS IMPLICATIONS FOR MANAGEMENT AND PREVENTION OF THE DISEASE

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Approximately 1 in 10 women develop breast cancer. Epidemiological studies have demonstrated that first-degree relatives of breast cancer cases are at twofold risk of developing the disease. Currently, three components of the genetic architecture of breast cancer have been delineated. (1) Rare, high penetrance (>10 fold) autosomal dominant cancer predisposition genes identified through linkage analyses. (2) Rare, intermediate penetrance (2–4 fold) susceptibility alleles discovered through large-scale sequencing of candidate genes. (3) Common, low penetrance (<1.5 fold) sus-

ceptibility alleles identified through genome-wide tag-SNP searches in breast cancer. We have been engaged in studies to identify variants in the latter two categories through Era of Hope funding. In particular, we have pioneered large-scale resequencing of candidate genes in case-control series to identify four DNA repair genes, *ATM*, *CHEK2*, *BRIP1*, and *PALB2* that act as intermediate penetrance breast cancer predisposition genes. These susceptibility genes are similar to *BRCA1* and *BRCA2* as they are characterized by multiple, individually rare, monoallelic, truncating mutations but they are associated with smaller increases in risk, approximately doubling the risk of breast cancer. All four genes encode proteins that function in DNA repair pathways and biallelic mutations in three of them (*ATM*, *BRIP1*, and *PALB2*) cause childhood developmental disorders associated with high risks of childhood cancer, similar to biallelic *BRCA2* mutations. Our current understanding of breast cancer indicates that multiple different genetic variants interact to give rise to most familial clusters and are likely contributing to a considerable proportion of nonfamilial cases. We, and others, are currently investigating how the new generation of breast cancer susceptibility alleles interact with each other and such analyses will be critical to risk estimation for individual women. Early results indicate that low penetrance variants interact multiplicatively with each other and some interact with *BRCA2* mutations to significantly alter the risk of breast cancer in high-risk families. Conversely, the known intermediate penetrance alleles do not appear to interact with *BRCA1/BRCA2* mutations presumably because they subvert DNA repair pathways that are already abrogated in *BRCA1/2* mutation carriers. Clinical implementation of low and intermediate penetrance susceptibility variants is complex and will need to be undertaken cautiously. However, the recent explosion of new breast cancer susceptibility variants provide promising new avenues for the identification of women at risk of breast cancer and for optimization of their management.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0204 and Cancer Research United Kingdom.

PI-15: SECOND HARMONIC PROPERTIES OF BREAST TUMOR COLLAGEN: DETERMINING THE STRUCTURAL RELATIONSHIP BETWEEN TUMOR STROMA AND HEALTHY STROMA

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The tumor stroma consists of the nonmalignant cells in a tumor, the basement membrane, and the extracellular matrix (ECM) and is known to play a significant role in tumor growth. Phenotypic alterations in stromal cells surrounding the malignant cancer cells, especially fibroblasts, modify the production and degradation of components of the ECM, resulting in a "reactive stroma" that is a hallmark of the neoplastic transformation. This highly altered ECM in turn is believed to play an active role in tumor progression and is known to affect tumor cell migration, to modify the availability of growth factors, and to directly signal tumor cells through integrins. As a result the character of the tumor ECM is a highly useful prognostic factor: In pathology practice, breast carcinomas are graded based on the structure and density of ECM staining while the presence of fibrotic foci in invasive ductal carcinoma of the breast indicates poor prognosis. Furthermore, elevated serum markers of collagen synthesis and degradation are indicators of breast cancer aggressiveness and poor patient survival.

The central role that the reactive stroma plays in tumor growth, progression, and metastasis means that there is a significant interest in understanding the reactive stroma itself, as well as the relationship between normal stroma and reactive tumor stroma. Second harmonic generation (SHG) has proven to be a useful window into the amount and organization of fibrillar collagen in biological tissues due to its relative specificity and the fact that it is an intrinsic signal. As part of an overall BCRP-funded project to investigate angiogenesis in living breast tumor models in animals, we explored the use of SHG to study the breast tumor ECM. This work, originally begun as a search for an optical method to recognize photodamage during vessel permeability studies, has provided unexpected insights into the fundamental nature of the fibrillar collagen in the breast tumor ECM and its relationship to the ECM of the healthy mammary gland. Specifically, we used polarization as well as scattering directionality of the SHG signal emitted from collagen fibers to quantify the radial extent of ordering of collagen triple helices, as well as the characteristic angular assembly of these triple helices. In spite of the extensive differences in collagen synthesis and degradation machinery, these optical techniques revealed that the collagen in breast tumor ECM is assembled with the same characteristic angle and the same radial extent of ordering as in healthy ECM. This suggests that the fibrillar collagen in breast tumors is somehow protected from the altered degradative environment of the reactive stroma, and that measurable serum markers of collagen synthesis and degradation that indicate breast cancer aggressiveness are produced via synthesis and degradation of a disordered, unprotected pool of nonfibrillar collagen.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0396.

PI-16: ABSTRACT WITHDRAWN

PI-17: USE OF EXOGENOUS PROGESTINS AND RISK OF IN SITU AND INVASIVE BREAST CANCER

Christopher I. Li
Fred Hutchinson Cancer Research Center

Background: There is strong evidence from both the Women's Health Initiative (WHI) randomized trials and observational studies that use of exogenous progestins plays an important role in breast cancer risk. Specifically, WHI found that use of estrogen plus progestin therapy increases breast cancer risk (hazard ratio [HR]=1.24; 95% confidence interval [CI]: 1.02–1.50) but that use of unopposed estrogen therapy does not (HR=0.77; 95% CI: 0.59–1.01). However, compared to our knowledge of the mechanisms underlying the association between estrogen and breast cancer, very little is known about the mechanisms underlying the relationship between progesterone and breast cancer. We propose two projects aimed at furthering our understanding of the relationship between progestin use and breast cancer risk.

Study Design:

Project 1: Using a case-control study design, we will compare 225 women aged 20–44 who have recently been diagnosed with in situ breast cancer to 1,000 population-based controls. In addition to ascertaining data on breast cancer risk factors through an in-person interview, tumor tissue and blood specimens will be collected for molecular analyses.

Project 2: This project will expand upon a completed case-control study that enrolled 469 controls, 601 lobular cases, and 442 ductal cases with an additional 450 subjects in each group. In addition to ascertaining data on breast cancer risk factors, tumor tissue and blood specimens will be collected for molecular analyses.

Specific Aims:

Project 1:

1. To determine if depo-medroxyprogesterone acetate (DMPA) use is associated with an increased risk of in situ breast cancer in premenopausal women.
2. To assess if other known breast cancer risk factors are related to risk of in situ carcinoma among premenopausal women and if they modify the association between DMPA use and in situ breast cancer risk.
3. To evaluate how DMPA is related to different types of in situ breast cancer.

Project 2:

1. How are known breast cancer risk factors, including hormone therapy, reproductive factors, anthropometric measures, alcohol use, and family history of breast cancer, related to risks of lobular and ductal carcinomas, and how do they interact with each other to alter these risks?
2. How do lobular carcinomas differ from ductal carcinomas in their expression of various tumor markers?
3. How do known breast cancer risk factors influence the expression of various tumor markers in lobular and ductal carcinomas?

Relevance: Given the large number of women exposed to progestins through either contraceptives or menopausal hormone therapies, clarifying the etiologic role of progestin in relation to breast cancer is of public health importance. While use of exogenous hormones has been the subject of numerous epidemiologic studies, few have evaluated DMPA use and few have evaluated risks by breast cancer type. The studies proposed will further our understanding of the potential risk of breast cancer associated with progestin use. A strength of both projects is that they include significant laboratory components that will enable biologic mechanisms underlying the possible association between use of different types of progestin and breast cancer risk to be assessed.

Study Progress: We are currently enrolling participants into both projects but have not yet accrued sufficient numbers of women to begin preliminary data analyses.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0482.

PI-18: UNDERSTANDING THE TUMOR SUPPRESSION FUNCTION OF BRCA1

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The maintenance of genomic integrity following DNA damage depends on the coordination of DNA repair with cell cycle checkpoint controls. A key component involved in DNA damage response is breast cancer tumor suppressor BRCA1.

In the past few years, we used genetics, cell biology, and biochemical approaches to elucidate the regulation of BRCA1 in DNA damage signaling pathways.

BRCA1 contains tandem BRCA1 C-terminal (BRCT) motifs. While human genetics studies convincingly demonstrate that the BRCT domains are important for the tumor suppressor function of BRCA1, the biochemical activities of these domains remained unknown until our study showing that BRCT domain is a phospho-protein binding

motif. We have demonstrated that the BRCA1 BRCT domain directly recognizes phosphorylated BACH1, a BRCA1-associated DNA helicase. The specific interaction between BRCA1 and phosphorylated BACH1 is cell cycle-regulated and is required during the S to G2 transition following DNA damage. Following up these observations, we have shown that BRCA1 can also interact with another binding partner, CtIP, in a phosphorylation-dependent manner, which is required for G2/M transition. Therefore, BRCA1 may form distinct complexes with different binding partners through its BRCT domains and thus participate in a variety of cell cycle and checkpoint responses.

The phospho-peptide binding activity of BRCA1 is also required for its localization to the sites of DNA damage, although this localization of BRCA1 is independent of the two known BRCA1 BRCT binding proteins BACH1 and CtIP. Using a biochemical purification approach, we recently identified a ubiquitin-binding protein RAP80 as a novel BRCA1-associated protein. RAP80 does not bind to BRCA1 directly, it forms a stable complex with a coiled-coil protein CCDC98. CCDC98 contains a consensus BRCA1 BRCT binding motif that is phosphorylated in vivo and interacts directly with BRCA1 BRCT domains. We have shown that both RAP80 and CCDC98 act upstream of BRCA1 in DNA damage response and are required for the recruitment of BRCA1 to the sites of DNA breaks.

More recently, we further defined how RAP80 itself is recruited to DNA damage foci. The ubiquitin-binding motifs of RAP80 are important for its focus localization. We have identified a new E3 ubiquitin ligase RNF8 that functions upstream of RAP80 and BRCA1 in DNA damage response. Based on this series of studies, we delineated a pathway from RNF8 to RAP80/CCDC98 to BRCA1 that is important for DNA damage checkpoint control.

Together, these studies have advanced our thinking mechanistically of how BRCA1 functions as a tumor suppressor. The future direction is to understand at molecular details how BRCA1 regulates DNA repair and facilitates the maintenance of genomic stability.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0470 and National Institutes of Health.

P1-19: FUNCTIONS FOR Wnt SIGNALING DURING MAMMARY TUMOR DEVELOPMENT

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Background: Ectopic Wnt signaling is oncogenic for mammalian epithelia and has been identified as the most common initiating mutation during the development of human carcinomas. Why is it so dangerous? We hypothesize that the key to understanding the oncogenicity of this pathway is to examine the cellular specificity of Wnt-dependent responses.

Methods: Genetically engineered mice were used to study the effect of gain and loss of function of Wnt signaling on stem cell activity and number and on the incidence of tumorigenesis.

Results: We have found that one of the Wnt signaling receptors is essential to the maintenance of normal breast stem cells but has no effect on the survival of more differentiated mammary epithelial cell (MEC) types. Despite the apparent lack of effect on the differentiated population, we find that without sufficient stem cell activity, the cells are relatively aged and their growth potential is lower. The cell fraction that expresses this receptor is greatly enriched in ductal stem cell activity, suggesting that this may be a functional mammary stem cell biomarker. Overexpression of Wnt ligands in mammary tissue promotes the accumulation of stem cell activity and induces hyperplasia and over-growth of the ductal tree. In this background, solitary adenocarcinomas arise that comprise cells with the full differentiation potential of normal mammary tissue. In fact, we have found that the overexpression of Wnt ligands by MECs triggers a whole body response that cooperates with the transformed precursor cell to generate a highly stromalized tumor.

Conclusion: We propose that a stem cell substrate for Wnt signaling activity, together with the organismal response, are important during the initiation and progression of breast tumors.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0491 and National Cancer Institute (R01 90877).

P1-20: CD4+ T CELLS REGULATE MACROPHAGE PHENOTYPE AND FUNCTIONALLY CONTRIBUTE TO MAMMARY TUMOR DEVELOPMENT

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University of California, San Francisco

Background and Objectives: During cancer development, the environment in which young "tumor" cells develop determines their ability to progress to the malignant state. Recent clinical and experimental evidence supports a critical link between

chronic inflammation and development of breast cancer. Historically, leukocytes found in and around developing tumors were thought to represent an attempt of the host to eradicate transformed neoplastic cells; however, recent epidemiologic and experimental evidence supports a promoting role for some immune cell types during cancer development. In neoplastic human breast tissue, macrophage, B and T lymphocyte presence increases during progression from pre-malignant in situ to malignant disease reflecting their potential increased significance with regards to malignancy. The objective of our studies is to identify functionally significant immune cell types and/or immune-regulated molecules that potentiate breast carcinogenesis and determine to what degree breast cancer is susceptible to immunomodulation as a therapeutic approach.

Methodology: In the present study, we assessed the functional significance of infiltrating adaptive immune cells during development of mammary adenocarcinomas by intercrossing the MMTV-PyMT mouse mammary carcinoma model with B and T lymphocyte-deficient mice (RAG1^{-/-}), B cell-deficient mice (JH^{-/-}), and mice deficient for either or both CD4⁺ and/or CD8⁺ T cells.

Results: We found that while loss of B and T lymphocytes did not alter latency of primary tumor development, tumor burden, or tumor histopathology, pulmonary metastasis (>80%) and total metastatic tumor burden were significantly ($p < 0.01$) diminished in a CD4⁺ T lymphocyte-dependent manner. Genetic elimination of CD4⁺ T cells phenocopied the PyMT/RAG1^{-/-} phenotype, as well as correlated with decreased numbers of circulating PyMT⁺ tumor cells and diminished presence of M2 or alternatively activated macrophages present in primary tumors. Using an organotypic 3-dimensional co-culture model with primary PyMT⁺ mammary epithelial cells (MECs) and naïve or tumor associated macrophages and CD4⁺ T cells, we revealed that tumor-associated CD4⁺ T cells regulate macrophage behavior/phenotype (polarization), that in turn regulate malignant and invasive behaviors of MECs in an interleukin (IL) 4-dependent manner.

Conclusions: Together, these data indicate that chronic activation of CD4⁺ T lymphocytes regulates production of type 2 inflammatory cytokines such as IL-4, that in turn elicits pro-tumor (as opposed to cytotoxic) bioactivities in macrophages that then enhance malignant and metastatic programming of neoplastic mammary tissue.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0416.

P1-21: GENOMIC GAIN OF 8q22 ACTIVATES METADHERIN AND PROMOTES CHEMORESISTANT METASTASIS OF POOR-PROGNOSIS BREAST CANCER

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The progression of cancer from an abnormal outgrowth to a life-threatening metastatic tumor is accompanied by a myriad of genetic and epigenetic alterations accumulated along the way. The challenge of distinguishing crucial drivers of metastasis from thousands of bystander alterations remains a major bottleneck in our quest to conquer cancer. The turn of the century has witnessed the advent of two parallel, but individually incomplete, genomic approaches to unravel the genetic mystery of cancer metastasis. Comparative expression profiling analyses of cancer cell line variants with different metastasis potentials, often obtained by in vivo selection in animal models, have led to the identification of several metastasis genes. However, much work remains to be done to validate the clinical relevance of metastasis genes identified in animal model studies. As a second approach, gene expression profiling of human tumor specimens has enabled the identification of several poor-prognosis signatures that are predictive of recurrence and metastasis risk in human cancers. Although different poor-prognosis signatures have proven to be operationally interchangeable for class prediction purposes in the clinic, the lack of overlap between different poor-prognosis signatures has posed a major challenge for understanding the biological underpinnings of cancer progression and metastasis, thereby hindering the development of targeted therapeutics. Identifying metastasis genes critical for driving the progression of human breast cancer requires innovative strategies to synergize advances in both clinical and experimental metastasis studies.

Here we report an integrative strategy to identify genomic alterations that are clinically relevant and functionally significant for breast cancer progression. We designed and applied a computational algorithm to identify recurrent genomic alterations associated with poor-prognosis breast cancer. Experimental metastasis assays of five candidate metastasis genes in the recurrent 8q22 genomic gain identified Metadherin (MTDH) as a mediator of breast cancer metastasis. Functional characterization of MTDH in animal metastasis models and in vitro functional assays suggested that it promotes metastasis and chemoresistance of breast cancer by mediating the interaction of tumor cells with vascular endothelial cells. Inhibition of MTDH expression in breast cancer cells reduced their metastasis potential to lung and other organs and sensitized them to stress and chemotherapeutic agents. Expression profiling revealed a MTDH-regulated gene set that includes several genes involved in the regulation of chemosensitivity of cancer cells to a broad spectrum of antineoplastic agents. Among these genes, ALDH3A1 was further validated to play a functional role in MTDH-mediated chemoresistance. Genomic gain of 8q22 and the concurrent overexpression

of MTDH were observed in 30%–40% of human primary breast tumors and were associated with poor survival and a higher risk of metastatic progression. The prognostic significance of MTDH overexpression is independent of other clinicopathological factors, such as ER, PR, HER2, p53 status, and tumor size. Together, these results illustrate an integrative strategy to uncover metastasis genes with important prognostic as well as therapeutic values and establish MTDH as a major target for the prevention and treatment of chemoresistant metastasis.

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P1-22: DENDRITIC AND T CELL DYSREGULATION IN TUMOR-DRAINING LYMPH NODES PREDICT BREAST CANCER RECURRENCE

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Background: We recently showed that significant decreases in T cells and dendritic cells within tumor-draining lymph nodes (TDLNs) strongly predict recurrence in breast cancer. We found that immune profile from CD4 T cells and CD1a of the axillary lymph nodes (ALNs) was the strongest predictor of 5-year disease-free survival (DFS) in early-stage breast cancer, regardless of tumor invasion status.

Objectives: To further investigate changes in phenotype and biological function of immune cells within TDLNs and tumor and to relate these changes to clinical outcome, we performed detailed architectural pattern analysis of TDLNs from breast cancer patients with or without relapse.

Methods: TDLN samples were obtained from 29 breast cancer patients, ages 29–76 years, treated at Stanford University Medical Center between February 1997 and January 1999. Five-year clinical outcome data for these patients were available. Immunohistochemistry (IHC) staining was performed on 3 µm thick tissue sections cut from formalin-fixed, paraffin-embedded nodes. Antibodies used included anti-CD4, anti-CD8, anti-CD1a, anti-CD83, anti-granzyme B, anti-FOXP3, and anti-AE1/AE3. Four panels, each containing three chromogens plus counterstain, were created, and isotype-matched antibodies were used as negative controls. The samples were imaged by an automated whole-slide scanning approach, using the Nuance spectral imaging system (CRI, Woburn, Massachusetts). The resulting images were analyzed using spectral unmixing algorithms and our custom image analysis software.

Results: CD8 T cells increased in density with increasing proximity to infiltrating tumor; however, granzyme B staining demonstrated that CD8 T cells within the tumor focus expressed the least potentially cytotoxic phenotype. A significant percentage (97%) of CD4 T cells bordering the tumor focus highly expressed CD25, compared to 18% within the rest of the ALN. T regulatory cell phenotype of CD25^{high} cells was confirmed by FoxP3 staining. FoxP3 expression inversely correlated with dendritic cell maturation. Increase in T regulatory cells and an immature dendritic cell profile by CD83:CD1a ratio both inversely correlated with 5-year DFS. Importantly, these patterns were observed in tumor-free lymph nodes.

Conclusions: These findings suggest that breast cancer can alter draining lymph nodes by inhibiting functional CD8 T cell responses, increasing regulatory T cell populations, and inhibiting dendritic cell maturation; thereby, preventing an effective anti-tumor immune response. Taken together, our results show that CD8 functional status, CD4 regulatory T cells, and dendritic cell maturation within ALNs represent useful prognostic indicators for breast cancer.

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P1-23: IMPAIRMENT OF INTERFERON RESPONSE IN IMMUNE CELLS OF BREAST CANCER PATIENTS

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Background: Dysfunction of the immune system has been documented in patients with many types of cancer; however, the underlying mechanisms are not well understood. Efficient response to interferon (IFN) signaling is critical for survival and development of effector and memory functions in lymphocytes. We recently demonstrated that IFN-α signaling is impaired in peripheral blood lymphocytes from melanoma patients. To determine whether impaired IFN-α signaling also arises in immune cells from breast cancer patients and hence may be a mechanism of immune dysfunction common to cancer, we assessed the response of peripheral blood leukocytes from breast cancer patients and healthy controls to IFN-α and IFN-γ.

Methodology: Peripheral blood mononuclear cells (PBMCs) from breast cancer patients and healthy controls were stained with cell surface markers, then stimulated with IFN-α, IFN-γ, or left unstimulated. The cells were then fixed, permeabilized, and stained with anti-STAT1-pY701 antibody prior to flow cytometric analysis. Fold

induction of STAT1-pY701 levels in all IFN-treated cells was determined by the ratio of mean fluorescence intensity of STAT1-pY701 levels in stimulated to unstimulated cells.

Results: Preliminary observations show that the fold induction of STAT1-pY701 was reduced in response to IFN-α stimulation in T cells, B cells, and NK cells and to IFN-γ stimulation in B cells from breast cancer patients (n=25) compared to age- and gender-matched healthy controls (n=13). The reduced fold change in STAT1-pY701 in response to IFN-α or -γ was observed equally in stage II, III, and IV breast cancer and was evident in patients before treatment and who had received adjuvant or neoadjuvant chemotherapy.

Conclusion: Defects in type-I and type-II interferon signaling in peripheral blood lymphocytes arises in breast cancer and melanoma. These may represent general cancer-associated mechanisms of immune dysfunction that contribute to disease progression and ineffective therapy. These findings may be used to design therapies to counteract immune dysfunction in breast cancer and to improve breast cancer immunotherapy.

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P1-24: UNDERSTANDING TUMOR-IMMUNE CELL INTERACTIONS AT THE SYSTEMS LEVEL USING GENE EXPRESSION PROFILING

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Preliminary studies in our laboratory demonstrated that the immune profile of tumor-draining lymph nodes (TDLNs) is of biological and clinical importance for patients with early-stage breast cancer and may provide powerful new prognostic tools. While lymph node metastasis is among the strongest predictors of disease-free and overall survival for breast cancer, the precise immunological nature of TDLNs is poorly understood. This project seeks to understand the biology of tumor-immune cell interactions in breast cancer, with particular interest on TDLNs. We propose that a complex interplay exists between the tumor cells and the host immune responses, which is a critical determinant in clinical outcome.

We took an integrative systems approach to study the dynamics between breast cancer and the immune responses by directly comparing the gene expression patterns between tumor, TDLNs, and peripheral blood. A number of studies have used microarray to profile breast tumor specimens, which represent heterogeneous cell populations consisting of tumor cells, stromal cells, and tumor-infiltrating immune cells. Our strategy was to profile purified tumor and immune cells, isolated from tumors and/or TDLNs. The initial set of gene expression data comprises 150 samples collected from 24 newly diagnosed breast cancer patients. These samples include immune cells and/or tumor cells from peripheral blood, breast tumor tissues, and TDLNs (tumor free or tumor involving). Of these, a total of 9 patient sample sets include tumor cells and their paired immune cells from tumor tissues, TDLNs, and blood to allow us to directly compare the gene expression patterns across three anatomical compartments. Live immune cells and tumor cells from each compartment were sorted from heterogeneous cell populations to high purity by flow cytometry, then analyzed by microarray technology.

Gene expression patterns of immune and tumor cells from each compartment are currently being analyzed at the systems level. Independent analysis of gene expression of purified tumor and immune cells provides important insights on the interactions between these cells within the tumor and TDLN milieu, and may elucidate mechanisms of immune dysfunction. These highly informative gene expression data are used to build in silico models focusing on gene networks and cross-talk between tumor and immune cells. This work may lead to novel diagnostic/prognostic tools to help predict clinical outcome and guide patient-tailored therapy in breast cancer. These results will be presented.

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P1-25: PROTEOMIC ANALYSIS OF BREAST CANCER AND SIGNALING

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Background and Objectives: It is now well recognized that there is a complex interplay between the malignant epithelial cells and the stromal cells in breast cancer. Several factors are exchanged between the carcinoma and stroma, which are necessary for the development and maintenance of the cancer. This includes secreted and membrane bound proteins that could play a key role in triggering signal transduction pathways in these cells. Thus, identifying both epithelial as well as stromal changes during early stages of breast tumorigenesis and correlating them with clinical follow up of patients could help predict tumor progression. Because phosphorylation is a

major post-translational modification in intracellular signaling events, characterization of kinase pathways and phosphoproteins can help us to define the pathways that are activated in malignant epithelial cells as well as in the cancer-associated stromal compartment.

Brief Description of Methodologies: We have taken a proteomic approach to identify both proteins as well as signal transduction pathways that are differentially expressed or activated in breast cancer associated epithelium or stroma. Tyrosine kinase pathways can be studied in a comprehensive fashion as antibodies for enrichment of tyrosine-phosphorylated proteins are readily available. Quantitative proteomic analyses can be carried out using *in vivo* or *in vitro* labeling methods. Using an *in vivo* quantitative mass spectrometry-based proteomic approach called SILAC (stable isotope labeling of amino acids in cell culture), that we have developed previously, we have carried out profiling tyrosine kinase signaling pathways in breast cancer. We have initially focused on HER2 signaling, as overexpression of HER2 is observed in a number of breast cancers. We are also carrying out studies using *in vitro* quantitative methodologies such as iTRAQ for a differential proteomic analysis of breast cancers.

Results to Date: Using cells that overexpress HER2 or cells derived from HER2-transgenic mice, we have been able to identify a number of signaling molecules that are specifically activated in response to HER2 signaling. Phosphoproteins identified included many signaling molecules that had not been previously linked to HER2, such as Stat1, Dok1, and delta-catenin. A number of previously uncharacterized HER2 signaling proteins such as Ax1 tyrosine kinase were also identified. Finally, we also identified a phosphorylation site in the HER2 receptor, Y877, located in the activation loop of the kinase domain that is distinct from the known C-terminal tail autophosphorylation sites. We are currently in the process of studying stromal fibroblasts in greater detail.

Conclusions: Our studies have the potential for identifying unique molecules and pathways that could either serve as biomarkers or therapeutic targets. In this regard, we have already identified a number of novel signaling molecules in HER2 signaling.

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P1-26: POTENTIAL ROLE OF PADI4 IN REGULATING THE ESTROGEN RESPONSE IN BREAST CANCER

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The estrogen signaling pathway is essential for normal mammary tissue development, and dysregulation of this pathway can lead to breast cancer. Estrogen action involves ligand-mediated activation of estrogen receptors (ER), which then bind directly to response elements on the promoter regions of target genes. Following binding, the ER recruits numerous primary and secondary coactivators, including protein arginine methyltransferases (PRMTs) and histone acetyltransferases (HATs). The PRMTs and HATs then facilitate gene activation by methylating and acetylating (respectively) histones leading to a more open chromatin configuration thus allowing core transcription factors to bind to the promoter.

Transcriptional downregulation of ER target genes is mediated, in part, by histone deacetylases (HDACs) that remove the "activating" acetyl marks from histones. However, the mechanism by which protein arginine methyltransferase (PRMT)-catalyzed histone arginine methylation (MeArg) is actively removed during downregulation is poorly understood. Peptidylarginine deiminases (PADIs) are a family of calcium-dependent enzymes that were previously shown to convert protein arginine to citrulline (Cit).

We have recently found that PADI4 also targets histone methylarginine residues for citrullination and have been testing the hypothesis that PADI4 may function as an ER transcriptional repressor via citrullination of either histone arginine or methylarginine residues at ER target genes. In support of this prediction, we have found that, in MCF7 breast cancer cells, conversion of histone H4MeArg3 to H4Cit3 on the estrogen-responsive pS2 gene correlates with recruitment of PADI4 to the promoter. Further, we found that depletion of PADI4 from MCF7 cells results in a strong upregulation of several ER target genes.

Interestingly, we have also found that the phenotype of PADI4-depleted cells is distinct from wild-type MCF7 cells in a number of ways. For example, at the morphological level, many PADI4-depleted cells appear to have converted from an epithelial phenotype to a more fibroblast-like mesenchymal phenotype. At the molecular level, this transition is supported by the finding that PADI4-depleted cells show a reduction in levels of epithelial markers such as E-cadherin and β -catenin and an increase in levels of mesenchymal markers such as vimentin. Additionally, we also found that depletion of PADI4 from MCF7 cells results in a greater than two-fold increase in the rate of cellular proliferation.

In sum, our results suggest that PADI4 may function as an estrogen receptor corepressor in breast cancer cells via citrullination of histone arginine and methylarginine residues' estrogen-responsive promoters. Further, our findings also suggest that PADI4 may also play a direct role in tumor suppression by blocking the epithelial to mesenchymal transition.

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P1-27: NANOTECHNOLOGY-ENABLED OPTICAL MOLECULAR IMAGING OF CANCER

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In this abstract we describe progress during the first year of an Era of Hope Scholar Award focused on the development of miniaturized photonics-based imaging technologies and complementary nanoscale molecular-targeted imaging agents that together have the capability to provide a significant new approach to molecular imaging of breast cancer. While there have been numerous prior published studies using endogenous reflectance and fluorescence spectroscopy for a variety of breast cancer screening, diagnostic, and monitoring applications, there have been far fewer attempts to develop microendoscopes that enable direct visualization of subcellular morphology and no attempts to date to develop a system for simultaneous high-resolution imaging and spectroscopy for breast cancer applications, which is one of the aims of our project. Our poster will provide proof-of-principle data demonstrating the capability for imaging fresh, intact unstained tissue with subcellular resolution using our first generation <1 mm diameter optical fiber probe in which each micron diameter fiber is used as a resolution element. Image processing approaches to remove fiber pixilation effects will also be described. In addition, we will discuss robust new synthesis and bioconjugation approaches for development of NIR scattering and emissive nanomaterials designed to be used in combination with our optical imaging systems. It is our ultimate goal to develop a simple, inexpensive, needle-compatible, fiber-integrated spectroscopy and imaging system capable of detecting both endogenous biochemical and morphological optical signals and exogenous optical signals created via bright nanoengineered molecular imaging agents. While significant effort has been devoted by the nanotechnology community to developing nanoparticles with progressively more favorable properties for imaging through optimizing "brightness" measured as scatter, absorption, or luminescence, it is our belief that in many cases, optical system design parameters and nanoparticle structure must be simultaneously optimized to achieve optimal imaging results. This is particularly true for the shaped gold nanoparticles being used in this Era of Hope project that have complex angular scattering patterns. Thus, in addition to initial imaging results using the system built in year 1 of our project, we will present additional data demonstrating the dramatic influence optical imaging geometry can have on sensitivity to targeted nanoparticles in tissue. By optimizing geometry, it is possible to obtain equivalent optical contrast using significantly lower nanoparticle dose potentially increasing the clinical viability of nanoparticle-based optical molecular imaging strategies.

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P1-28: HYBRID NANOPARTICLES FOR TARGETING CANCER

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Co-administration of chemotherapeutic agents with drugs that inhibit tumor angiogenesis has emerged as an attractive therapeutic strategy in cancer. However, chronic vascular shutdown can preclude the uptake of the cytotoxic agent into the tumor, especially in the later cycles of chemotherapy. In a recent study, we engineered a multifunctional nanoparticle, called a nanocell, which could address these problems by spatiotemporally releasing an antiangiogenesis and a chemotherapeutic agent following focal entrapment within the tumor. Recent evidence has, however, indicated that antiangiogenesis therapy can lead to "reactive resistance" through the upregulation of a hypoxia-driven growth factor autocrine loop that can lead to tumor dormancy with an increase in tumor invasiveness. Interestingly, the survival, proliferation, and invasiveness of both the angiogenic endothelial cells and the hypoxic tumor cells are exquisitely controlled by a diverse set of growth factors that converge into the same downstream signaling cascades of which the phosphatidylinositol 3 kinase or mitogen-activated protein kinases are the most widely implicated in tumorigenesis and angiogenesis. Our goal is to engineer biological mechanism-inspired multifunctional nanoparticles that can enable spatiotemporal targeting of convergent points between aberrant signaling pathways within a tumor and thereby predispose the tumor to the effects of cytotoxic agents and additionally avoid global adverse effects. We have developed a coculture *in vitro* breast cancer system that allows us to test for drug combinations that are optimized toward targeting different tumoral compartments. Using this system, we demonstrate that inhibition of MAPK and PI3K predisposes the tumor to the cytotoxic effects of chemotherapy. Furthermore, we have engineered a prototype hybrid nanoparticle that can spatiotemporally deliver the inhibitors to the

different tumoral compartments thereby predisposing the tumor to the effects of the cytotoxic agents.

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P1-29: CORRELATION OF UMBILICAL CORD BLOOD HEMATOPOIETIC STEM AND PROGENITOR CELL LEVELS WITH BIRTH WEIGHT AND THE PRENATAL ORIGIN OF CANCER

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Background: The levels of in utero/perinatal mitogens and other factors determine the size of the stem cell pool in the developing fetus; elevated tissue stem cell numbers drive the formation of larger organs and hence might be associated with larger birth weights. The greater the stem cell pool size, however, the greater the chance that one of the stem cells will be mutated by a carcinogen or undergo a DNA replicative error, initiating oncogenic transformation. Hence, individuals with high birth weights might be at greater lifetime cancer risk. Indeed, birth weight has been reported to be positively associated with the risk for breast cancer, and perhaps other forms of cancer, in adult life.

Multiple linear regression analysis on the association of hematopoietic stem cells with birth weight									
Analytic model	Cell measurements	Birth weight (g) in categories						Birth weight per 500g	
		<3,000		3,000-3,499		3,500-3,999		>=4,000	
		% difference (95% CI)	Reference (geometric mean*)	% difference (95% CI)	% difference (95% CI)	% difference (95% CI)	% difference (95% CI)	% difference (95% CI)	% difference (95% CI)
Adjusted for core covariates**	TNC	2.1 (-8.5, 14.0)	0.0 (15.00)	7.8 (-1.7, 18.3)	8.4 (-8.1, 27.8)	3.0 (-2.5, 8.9)			
	MNC	5.7 (-6.4, 19.3)	0.0 (6.91)	6.5 (-3.9, 18.0)	12.5 (-6.2, 34.9)	2.1 (-3.8, 8.4)			
	CD34 ⁺	2.4 (-19.2, 26.7)	0.0 (7.04)	12.5 (-8.1, 37.7)	20.8 (-15.5, 72.8)	9.6 (-2.6, 23.2)			
	CD34 ⁺ CD38 ⁺	-1.1 (-23.6, 28.1)	0.0 (3.11)	19.1 (-4.3, 48.1)	47.9 (0.4, 117.8)	18.6 (1.6, 31.2)			
	CD34 ⁺ c-kit ⁺	13.7 (-14.0, 50.4)	0.0 (5.80)	18.9 (-5.5, 49.6)	34.2 (-10.4, 101.1)	10.0 (-4.0, 26.1)			
	CFU-GM	37.0 (2.1, 85.8)	0.0 (4.04)	29.0 (-0.1, 66.6)	31.4 (-16.2, 106.1)	6.1 (-8.4, 23.0)			
Adjusted for core covariates and IGF-1	TNC	0.6 (-10.0, 12.5)	0.0 (15.00)	9.7 (-0.4, 20.8)	12.3 (-5.5, 33.4)	4.5 (-1.5, 11.0)			
	MNC	4.0 (-8.0, 17.7)	0.0 (6.91)	8.5 (-2.5, 20.6)	16.8 (-3.5, 41.4)	3.7 (-2.9, 10.8)			
	CD34 ⁺	11.0 (-12.3, 40.6)	0.0 (7.04)	2.5 (-16.5, 25.8)	-0.3 (-31.0, 44.0)	0.6 (-11.4, 14.2)			
	CD34 ⁺ CD38 ⁺	5.2 (-19.0, 38.6)	0.0 (3.11)	10.9 (-11.5, 38.8)	27.6 (-14.7, 90.9)	7.9 (-6.2, 24.0)			
	CD34 ⁺ c-kit ⁺	22.8 (-6.9, 62.1)	0.0 (5.80)	7.0 (-15.3, 35.2)	10.5 (-26.9, 66.9)	0.7 (-12.9, 16.4)			
	CFU-GM	45.6 (8.6, 95.1)	0.0 (4.04)	15.5 (-11.8, 50.9)	11.5 (-29.8, 77.1)	-1.7 (-16.2, 15.3)			

*Unadjusted geometric means. TNC, total nucleated cells x 10⁶/mL; MNC, total mononuclear cells x 10⁶/mL; the unit for the stem cell populations (CD34⁺, CD34⁺CD38⁺, CD34⁺c-kit⁺, and CFU-GM) was 1/1,000 MNC. **Core covariates included mother's age, race of parents (both Caucasian or not), parity, gestation duration, gender of baby (male or female), delivery time (night or day).

Methods: We measured the umbilical cord blood concentrations of hematopoietic stem and progenitor populations in 288 singleton infants to determine whether or not these measurable cells, serving as surrogates of overall stem cell potential, were positively associated with birth weight. Multivariate linear regression was used to examine the association between natural log-transformed measures of stem cell potential (dependent variable) and birth weight (independent categorical or, alternatively, continuous variable, adjusting for maternal and neonatal characteristics). Levels of IGF-1, which among hormones have been reported to have the strongest association with levels of stem cells, were further adjusted for to explore the influence on the association between birth weight and stem cell measurements.

Results: Across the whole range of birth weight, there was a positive relation between birth weight and CD34⁺CD38⁺ cells with a 500 g increase in birth weight being associated with a 15.5% higher cell concentration (95% confidence interval: 1.6% to 31.3%). CD34⁺ and CD34⁺c-kit⁺ cells had J-shaped relations, and CFU-GM cells had a U-shaped relation with birth weight. Among newborns with ≥3,000 g birth weights, concentrations of these cells increased with birth weight while those below 3,000 g had higher stem cell concentrations than the reference category of 3,000–3,499 g. Adjustment for cord blood plasma insulin-like growth factor-1 (IGF-1) levels weakened the association of birth weight with stem and progenitor cells.

Conclusions: The J-shaped relation observed between birth weight and stem cell measurements requires further confirmation while the positive associations between birth weight and stem cell measurements for term newborns with a normal or higher birth weight support a role of the intrauterine environment in the etiology of breast and perhaps other forms of cancer.

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P1-30: ACTIVATABLE CELL-PENETRATING PEPTIDES FOR IMAGING TUMORS

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We are synthesizing and testing novel imaging agents based on activatable cell-penetrating peptides (ACPPs) that are substrates for matrix metalloproteinases (MMPs), proteases highly expressed in the tumor microenvironment. A simple ACPP is glu₉-[MMP-cleavable linker]-arg₉-Cy5, in which the polyanionic glu₉ prevents the polycationic arg₉ cell-penetrating peptide (CPP) from sticking to and entering cells. Proteolysis of the linker separates the glu₉ from the arg₉, freeing the arg₉-Cy5 to be taken up onto and into cells in the vicinity of the protease activity, where the Cy5 can be visualized by its far-red fluorescence. Such ACPPs give useful in vivo fluorescence contrast, peaking a few hours after intravenous injection, in xenografts of various human tumor cells into nude mice as well as in MMTV-polyoma-middle-T (PyMT) mammary tumors in immunocompetent transgenic mice. This fluorescence contrast looks promising as a real-time guide to surgical resection.

Thus Figure 1 shows that small GFP-expressing residual tumors that are invisible by ordinary color images or even green fluorescence can be detected and excised with the help of red ACPP fluorescence. Attachment of the peptides to macromolecular carriers such as dextran, albumin, or dendrimers gives better contrast but slower pharmacokinetics. ACPP-containing nanoparticles in which Cy5 is replaced by Gd chelates constitute smart magnetic resonance (MR) contrast agents, which highlight PyMT tumors or downstream lymph nodes in T₁-weighted MR images.

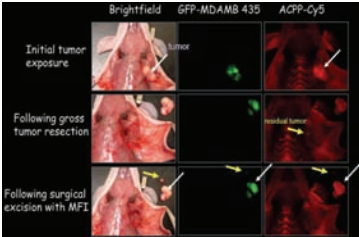
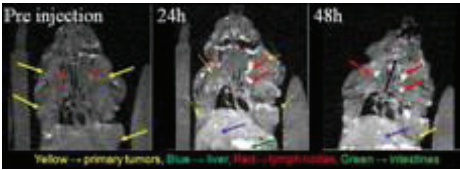


Figure 1. Surgical excision of tumor and mets is more complete with ACPP fluorescence guidance

Figure 2 shows ACPP accumulation in metastatic lymph nodes draining PyMT tumors, imaged at 7 Tesla. Thus ACPPs offer a promising new general strategy to detect protease activities in vivo and to concentrate imaging and perhaps chemotherapeutic agents within tumors for diagnosis, intraoperative localization, and treatment although much optimization is still required.



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P1-31: A HORMONAL APPROACH TO THE CHEMOPREVENTION OF BREAST CANCER

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The aim of this research is to develop a hormonal chemopreventive approach to achieve the protective effect against breast cancer of an early full-term pregnancy. Mammographic densities (MDs) are permanently reduced by births; this relationship is being explored to determine if this is an important part of the mechanism by which births provide protection. This work is being conducted both in humans and in rodents (UPenn).

Rat: Rats have been treated with estradiol (E₂), progesterone (P₄), E₂+P₄, β-HCG, or perphenazine. All except P₄ showed mammary epithelial "differentiation" comparable to that of parous animals. E₂, E₂+P₄, and β-HCG were protective against MNU-induced mammary tumors, P₄ was not; perphenazine was partially protective. Protection with lower "dose" E₂+P₄ is under investigation.

Breast expression profiling in parous and nulliparous rats from 4 strains showing E₂+P₄ protection yielded a common gene signature [1]. Comparison is ongoing with the hormonal treatments.

Women: Normal breast samples (n=90 to date) from women undergoing mammaryplasty are being studied for gene expression. Breast samples (n=32 to date) are also been collected early in pregnancy (needle biopsy taken within 10 min of a termination) and within a year thereafter to find out whether the lower E₂ and P₄ levels of early compared to late pregnancy induce the "protective" gene changes.

Progesterone Receptor: Work in the rat [2] suggested that breast PRA and PRB expression may distinguish a parous from a nulliparous breast. However, no evidence of such was found in women. The effects of pregnancy were striking. PRA expression decreased from 21% to 4% in pregnant subjects while PRB expression increased from 54% to 96%.

MDs: The major constituent of MDs is collagen, and almost all breast epithelium is surrounded by dense collagen [3]. The extent of MDs may simply be a surrogate of the amount of breast epithelium. We have now provided support for this interpretation by detecting collagen-encoding RNAs both in breast epithelium and fibroblasts and are currently evaluating this further.

High-Dose Progestin Treatment: This study obtains pre- and posttreatment breast needle biopsies from women receiving a high-dose progestin (approaching pregnancy levels) for endometrial hyperplasia.

Progestin Dose in Oral Contraceptives (OCs): This study obtains breast needle biopsies from women on one or other of 2 OCs with the same estrogen dose but 1 and 0.4 mg norethindrone as progestin. Women seeking an OC are recruited and have a breast biopsy taken after 3 months of OC use. This protocol is aimed at demonstrating that a lower progestin dose OC has a lower breast proliferative effect and may thus reduce the risk of breast cancer noted with current and recent past use of standard OCs [4] as our overview of menopausal hormone therapy predicts [5].

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P1-32: EARLY DETECTION OF METASTASIS-PRONE BREAST CANCERS

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Cancer screening programs are intended to decrease cancer-related mortality by detecting and treating cancer at an earlier stage than would have happened in the absence of screening and/or by identifying and removing preneoplastic lesions thereby preventing the development of cancer. The success of this approach is evident in both colon cancer and cervical cancer. The results achieved through mammographic screening are less positive. The widespread introduction of population-based screening mammography programs in the United States beginning in the mid 1980s resulted in a dramatic rise in the detection of stage I cancers as well as ductal carcinoma in situ. However, the expected concomitant decrease in the incidence rates of regional cancers has not occurred, and the relative change in breast cancer mortality attributable to screening is less than that seen in colon and cervical cancer. These data strongly suggest that current mammographic screening strategies are not detecting the lesions that are precursors to metastasis-prone breast cancers sufficiently early to allow curative local treatment.

The overall goal of this project is to develop anatomical imaging strategies that are sufficiently sensitive and specific to detect metastasis-prone cancer precursor lesions before they have metastasized and molecular histopathology procedures that will reveal the true extents of cancers as a guide to more complete surgical resection. We are working to improve anatomic localization by developing MRI and/or PET imaging agents based on macromolecule platforms that are specifically targeted to metastasis-prone basal or luminal/amplifier tumor subtypes and by developing digital imaging algorithms that recognize metastasis-prone lesions in digital mammograms. In addition, we are working to improve histopathological analysis by using scanned ion beam mass spectrometry to image tissue sections stained with mass-tag-labeled affinity

reagents that bind to metastasis-prone basal or luminal/amplifier cells. We envision that these molecularly targeted anatomic and histopathological imaging procedures will contribute to the development of a personalized breast cancer management strategy in which high-throughput screening strategies are employed to identify individuals at high risk of cancer, for example, using high volumetric breast density analysis or blood serum screening. High-risk individuals identified in the first screen will then be assessed using imaging technologies to anatomically localize metastasis-prone precursor cancers based on their molecular characteristics. Once a suspicious lesion is detected, molecular histopathology will be used to guide its surgical removal. Finally, therapeutic strategies targeted specifically to metastasis-prone disease will be used to treat any residual tumor or disseminated disease that may still remain. We report now on progress on the development of the molecular contrast reagents and imaging strategies needed to support these imaging strategies.

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P1-33: TOWARD A PROPHYLACTIC VACCINE FOR BREAST CANCER

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Our goal is to create a vaccine that can be given to healthy adults to prevent breast cancer. The premise for this concept is based on the fact that tumors produce many protein variants that are foreign to the immune system. If some of these variants occur in nascent breast tumors in many individuals, they can be used to vaccinate women to prevent cancer as is done for infectious disease. We have shown in mouse models that in principle this concept works. The question now is whether enough of such protein variants is frequent enough in breast tumors to constitute such a vaccine.

We are searching for these variants with a number of approaches. The criteria for identifying these variants is that they be found frequently (>3%) in the breast tumor cell lines and tumor samples in our test panel and not at a significant level in normal cells. Additionally, the variant should be predicted to bind the HLA in a majority of humans. We have four basic approaches for searching.

Informatic: We are searching all existing and new databases of tumor cDNA and genomic sequences looking for frameshift and other forms of variant proteins. This includes the recent reports of extensive DNA sequencing of tumors. These are evaluated by sequencing the cDNA or genomic DNA in our breast tumor panel.

Proteomic: We have established a system to identify novo-peptides on tumor surfaces that are not present on normal cells. Peptides from tumor cells are scored against the databases of predicted foreign peptides we have created.

RNA Splice Variants: Mis-splicing events can often produce variant peptides. We are searching tumor cDNA for likely splice variants that would be tumor specific.

Immunological: Where there seems to be a mouse homolog of a variant tumor protein, we are directly screening for protection against challenge with tumor lines in the mouse. For most candidate peptides, we will screen in vitro with cytotoxic T-cell assays to determine if the variant peptide can induce killing of human breast tumor cells. Any candidate discovered by one approach is validated by at least one orthogonal test.

Funding for this project initiated in October 2007. A primary initial focus has been on a thorough search of both the literature and databases for tumor-specific translocations that would create new peptides. Two candidates have been found to date that are 2.5% and 25% frequent in the tumor panel. They would produce a frameshift peptide of 5 and 7 amino acids, respectively. Both candidates were discovered using an algorithm we developed to search the NCI EST databases for new translocations. At least 25 other candidates will be evaluated. The screen of the mis-splicing variants has proved difficult due to the much higher than expected variation in normal cells. However, at least one candidate is being pursued from this source. A database for the predicted potential variant proteins in tumors has been created. This is the basis for searching for non-normal peptides from the surface of tumor cells. This approach has produced a large number of candidates, the best of which are now being evaluated.

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BREAST CANCER CENTERS OF EXCELLENCE I

Poster Session P2

P2-1: INFLAMMATORY BREAST CANCER PATHOGENESIS MEDIATED BY TRANSLATION INITIATION FACTOR eIF4G OVEREXPRESSION AND UNORTHODOX PROTEIN SYNTHESIS

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Background: The molecular mechanisms that drive development of inflammatory breast cancer (IBC) are unknown. IBC is a particularly aggressive form of breast cancer. Despite an incidence of only 2% in the United States with a median survival of only 3 years, it accounts for 7% of deaths annually. Clinically, IBC presents typically as a stage III disease, and although it is often classified as a form of locally advanced breast cancer, our work and others indicate it is a genetically and molecularly unique form of breast cancer. IBC is characterized by intense angiogenic activity, involving a high level of endothelial cell proliferation and remarkably strong expression of pro-angiogenic factors, particularly VEGF-A. IBC is more likely to be estrogen and progesterone receptor negative and Her2 positive than other breast cancers. IBC is unusual in generation of tumor emboli that are highly metastatic cell clusters (emboli) that require minimal vascularization due to strong overexpression of VEGF-A that permeabilizes the vasculature and sustains emboli proliferation. IBC also uniquely overexpresses the adhesion molecule E-cadherin, which promotes metastasis by blocking adherence of IBC emboli to stroma. Using patient IBC specimens and development of modified IBC cell lines and animal models, we show that many of the extreme pathological disease characteristics of IBC, including VEGF-A and E-cadherin overexpression, result in large part from the genetic amplification and overexpression of a single gene encoding translation initiation factor eIF4G. eIF4G overexpression reprograms the protein synthetic machinery of IBC cells for increased translation of a small group of mRNAs that promote IBC tumor cell survival, vascular permeabilization/angiogenesis, and emboli formation.

Materials and Methods: Protein factor levels expressed in IBC tissues were determined by immunohistochemistry (IHC), and in IBC cell lines by immunoblot analysis. eIF4G expression was silenced by shRNA adenoviruses. To analyze tumor growth and vasculogenesis we used the chicken chorioallantoic membrane (CAM) model, and xenograft cell subcutaneous implantation in nude mice.

Results and Discussion: Gene expression and IHC studies found that >80% of IBC tumor specimens and the SUM149 IBC cell line strongly overexpressed eIF4G (5-fold) compared to normal tissue, with no alteration in other translation factors (n=42; p=0.0012). In animal models, shRNA silencing of eIF4G by ~90% only slightly reduced global protein synthesis, but selectively inhibited IBC growth, VEGF, p120 catenin and Bcl2 mRNA translation, as well as cell surface retention of E-cadherin, vasculature permeabilization and angiogenesis—all of the key hallmarks of IBC pathogenesis. E-cadherin is retained on the cell surface by interaction with p120 catenin. Engineered overexpression of p120 restored E-cadherin to the cell surface and in part IBC formation. Surprisingly, mutational analysis of VEGF-A and p120 catenin mRNAs showed they contain a cap-independent translation element known as an Internal Ribosome Entry Site (IRES), that required high levels of eIF4G for translation during hypoxia. These and other data demonstrate that high levels of eIF4G are singularly responsible for many of the unique pathologic properties of IBC. Inhibition of eIF4G might constitute an attractive target for development of new therapeutics for IBC.

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P2-2: GLUTATHIONE DEPLETION SENSITIZES HORMONE-INDEPENDENT HUMAN BREAST CANCER CELLS TO ESTROGEN-INDUCED APOPTOSIS

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Glutathione (GSH) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways as well as in the antioxidant system of aerobic cells. In many cell systems, pharmacological GSH depletion with the GSH biosynthesis inhibitor L-buthionine-S,R-sulfoximine (BSO) leads to cell death and highly sensitizes tumor cells to apoptosis induced by standard chemotherapeutic agents. Our laboratory has previously reported the development of a unique subclone of the MCF-7 human breast cancer cell line, named MCF-7:5C, which grows maximally in the absence of endogenous estrogen but undergoes apoptosis within 24–48 hours of 17 β -estradiol (E₂) treatment via activation of the mitochondrial cell death pathway (Lewis et al, *J. Natl. Cancer Inst.* 2005; 97:1746-59). In the present study, we have identified and characterized another subclone of the MCF-7 cell line, named MCF-7:2A, which unlike the MCF-7:5C cells, undergoes apoptosis only in the presence of E₂ plus BSO but not E₂ alone. Exposure of MCF-7:2A cells to 1 nM E₂ or 100 μ M BSO for 48–96 hours did not produce cell death; however, the combination treatment produced a dramatic increase (7-fold) in apoptosis, which was evidenced by

Annexin V-PI and TUNEL staining. Microarray studies revealed that glutathione synthetase (GSS) and glutathione peroxidase 2 (GPX2) genes were overexpressed by 6-fold and 40-fold, respectively, in MCF-7:2A cells compared to hormone-responsive MCF-7 cells. Cellular GSH levels were also significantly (p<0.001) elevated in MCF-7:2A cells compared to MCF-7 cells and BSO almost completely depleted GSH. Induction of apoptosis by the combination treatment of E₂ plus BSO was also evidenced by changes in Bcl-2, Bcl-xL, and Bax expression, mitochondrial membrane potential and cytochrome c release, poly(ADP-ribose)polymerase (PARP) cleavage, and caspase 9 and caspase-7 activation. The combination treatment also markedly reduced phosphorylated p38 MAPK and upregulated phosphorylated JNK levels in MCF-7:2A cells. In addition, blockade of the JNK pathway using the inhibitor SP600125 almost completely attenuated the apoptotic effect of E₂ plus BSO thus suggesting an important role for JNK in mediating the apoptotic effects of E₂ and BSO in MCF-7:2A cells. In conclusion, our data indicate that GSH participates in apoptosis in hormone-resistant breast cancer cells and that depletion of this molecule may be critical in predisposing these cells to apoptotic cell death.

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P2-3: RACIAL DISPARITIES IN THE INITIATION AND INTENSITY OF ADJUVANT THERAPY FOR BREAST CANCER

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Background: One of the most important issues in breast cancer research is the disparity in survival between black and white women with breast cancer. Many studies have shown that, even on a stage-for-stage basis, black women have a worse survival outcome that is quite significant (up to twofold). Some of this disparity may be due to an increased frequency of more aggressive breast cancer in black women (ER-negative, HER-2 *neu* positive, higher Ki-67). However, even after controlling for these factors, a significant disparity remains. Some studies suggest that black women are less likely to receive optimal systemic adjuvant therapy than white women and this may account for some of the survival disparities. Reasons for the black-white differences in receipt of optimal therapy are the subject of our Center.

Objective: To study the biological, toxicity-related, behavioral, communication, economic, psychosocial, cultural, and molecular genetic factors that may create barriers that lead to the non-receipt of optimal chemotherapy and hormonal therapy and may account for some of the disparities in cancer survival.

Methods: Women meeting study inclusion criteria are recruited and consented at 3 partner sites across the country – a consortium of 12 hospitals in New York City, Henry Ford Health System in Detroit, and Kaiser Permanente of Northern California. A series of telephone interviews are conducted over the telephone by the Columbia University research team. The baseline interview, approximately 45 minutes in duration, is conducted after definitive surgery prior to the administration of the third chemotherapy treatment if chemotherapy is to be administered, or within 12 weeks of surgery if no chemotherapy is to be administered. Follow-up surveys are administered 4–6 weeks later and 12–24 weeks after completion of the baseline interview and take approximately 15 minutes. Women who undergo hormonal therapy are asked to complete additional interviews every 6 months in the first 2 years following diagnosis and annually thereafter. A specimen (blood, buccal cells, or saliva) for DNA analysis is collected from each participant. Additionally, the medical record of each participant is reviewed and treating physicians will be surveyed.

Results: To date, 486 women with stages 1–3 breast cancer have been referred to the study and 367 (76%) were successfully consented. Of the 367 enrolled participants, 269 (73%) were recruited from Northern California, 82 (22%) from NYC and 16 (5%) from Detroit. Of those enrolled, 16% are black, 74% white, 4% Asian, 3% Hispanic, and 3% other. A total of 952 surveys have been completed – 337 baseline, 467 follow-up, and 148 hormonal therapy. At baseline, 39% of participants were stage 1, 31% stage 2, 7% stage 3, and 23% stage unknown (DCIS and stage 4 were excluded).

Conclusions: Our DOD Breast Cancer Center of Excellence has successfully begun recruitment. Our *a priori* goal is to recruit 20% black subjects and we are near that goal. We are also more than one-third toward our overall recruitment goal of 1,000 subjects. We achieved a 76% participation rate of referred patients, a respectable level of participation.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0399.

P2-4: PRELIMINARY DATA ON PATIENT DECISION-MAKING REGARDING THE INITIATION OF ADJUVANT THERAPY FOR BREAST CANCER

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Background: The observed racial disparity in breast cancer survival may stem in large part from differences in breast cancer treatment. Recently, an emphasis has been placed upon dose-intensity and dose density, suggesting that more intensive regimens with higher toxicities may provide even better success rates and improve survival for those who receive such regimens. Black women are less likely than white women to receive the optimal recommended doses or dose intensity of such regimens that may contribute to their worse survival. The patient's decision to initiate treatment for breast cancer is a complicated process, based on factors that include patient/physician communication, cognitive, affective and psychosocial/socioemotional factors surrounding breast cancer and its treatment, sources of social and emotional support, and the patient's desired level of involvement regarding her treatment.

Objective: To evaluate who is involved in the decision-making process for adjuvant breast cancer therapy among black and white women enrolled in a longitudinal study of racial disparities in breast cancer treatment.

Methods: Women were recruited and consented at 3 partner sites across the country – a consortium of 12 hospitals in New York City, Henry Ford Health System in Detroit, and Kaiser Permanente in Northern California. Baseline data were collected via telephone interview conducted by the Columbia University research team. The baseline interview, approximately 45 minutes in duration, was conducted after definitive surgery prior to the administration of the third chemotherapy treatment or within 12 weeks of surgery if no chemotherapy is to be administered.

Results: We report on baseline interviews conducted with the first 223 subjects. One third of women were recruited from New York and the remaining women from Northern California. In this sample, 94% were referred to a medical oncologist and 69% of women reported that chemotherapy was offered or recommended. Among women for whom chemotherapy was offered or recommended, 91% decided to undergo this treatment. With regard to decision-making, 2% of women preferred to have the doctor alone make the decision about treatment, 23% preferred the doctor make the decision but consider her opinion, 38% preferred to share the decision-making with the doctor equally, 34% preferred to make their own decision with input from the doctor, and 3% preferred to make the decision on their own, independent of the doctor's opinion. Results did not significantly vary by race, age, stage of disease, or geographic location. Others involved in the subject's decision-making process included husband/partner, family members, and friends and neighbors. White women were more likely than black women to involve the husband/partner (57% versus 32%, $p=.03$) and women ≥ 50 years were more likely than women <49 years to involve their children or grandchildren in the decision-making (20% versus 6%, $p=.03$).

Conclusions: Shared decision-making has become a reality for breast cancer treatment with some variability in the degree of physician input desired by breast cancer patients. Social support, as manifested by husband/partner and close family members, also plays a crucial role in helping women with breast cancer to reach these tough decisions. Future data collection will permit more detailed analyses of these issues.

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P2-5: HETEROGENEITY OF BREAST CANCER METASTASES: COMPARISON OF THERAPEUTIC TARGET EXPRESSION AND PROMOTER HYPERMETHYLATION BETWEEN PRIMARY TUMORS AND THEIR MULTIFOCAL METASTASES

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A comprehensive comparison of biomarker expression between patients' primary breast carcinoma (PBC) and their metastatic breast carcinomas (MBCs), as well as between different MBCs from different sites in the same patient, has not been performed.

We performed 15 rapid autopsies (median post-mortem interval, 3 hours) on consenting patients who died of MBC. Paraffin tissue blocks from the patients archived PBC and multiple different MBCs were used to construct single patient tissue microarrays

(TMAs). We present data on the first 10 cases. Ten TMA slides containing PBCs and in total 655 spots derived from 120 MBC sites were immunohistochemically labeled for the following: estrogen receptor (ER), progesterone receptor (PR), Her-2/neu, E-cadherin, Fascin, EGFR, MET, Cox-2, CK 5/6, and Mesothelin. Methylation of the *RASSF1a*, *HIN1*, *Cyclin D2*, *Twist*, *ER*, *E-cadherin*, and *RAR β* gene promoters was assessed quantitatively on dissected PBC and MBC samples. Extensive heterogeneity was observed between PBC and their paired MBC, as well as among multiple MBC from the same patient. The patterns observed are summarized into three categories:

1. **Markers Downregulated Uniformly in All Metastases of a Case:** ER, PR. Four cases were ER-PR- and 3 cases were ER+PR+ in the PBC and all MBC. However, 1 ER+PR+ PBC was ER-PR- in all its MBC, 1 ER+PR- PBC was ER-PR- in all its MBC, and 1 ER+PR+ PBC was ER+PR- in all its MBC.
2. **Markers Consistently Expressed between Primary and Metastasis:** Fascin, implicated in the lung metastasis gene expression signature of PBC (*Nature* 2005; 436: 518-524), was overexpressed in the PBC and all MBC in 1 of 10 cases. Interestingly, this was the only case with bulky lung metastasis. Promoter hypermethylation of *RASSF1a*, *HIN1*, *Cyclin D2*, *Twist*, *ER*, *E-cadherin*, and *RAR β* was overall very similar in the PBC and all MBC in all 10 evaluable cases.
3. **Markers Variably Expressed among Metastases:** E-cadherin was variably down-regulated in the MBC of one case; the E-cadherin positive invasive ductal PBC gave rise to both E-cadherin positive ductal MBC and E-cadherin negative MBC with lobular morphology.

Variable overexpression in MBC compared to the PBC was observed for Cox-2 (5 cases), EGFR (4 cases), MET (4 cases), and Mesothelin (4 cases). No case strongly overexpressed Her-2/neu by IHC, but 3 cases showed variable expression ranging from negative to weakly positive (2+) in different MBC. In one case, variable low-level Her-2/neu amplification was found. Interestingly, the EGFR and MET overexpressions were restricted to ER-/PR- cases that were CK5/6+ and Her-2/neu negative, and hence qualify as basal-like cancers. In one case, EGFR was strikingly and uniformly overexpressed in metastases compared to the primary tumor, but this did not correlate with EGFR gene amplification. Therefore, we conclude that therapeutic targets identified in the PBC or even some MBC may or may not reflect targets present in all metastatic sites, depending upon the specific target in question.

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P2-6: ADVOCATE MENTOR PROGRAM: EDUCATING ADVOCATES ABOUT GENOMICS

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Background: The Research Advocacy Network (RAN) was formed in 2003 to bring together participants in the research process with the focus on educating, supporting, and connecting patient advocates with the research community. The patient advocacy movement has been instrumental in doubling NIH funding, lobbying federal and state governments for insurance coverage, routine patient care costs in clinical trials, new drugs, and treatment appeals mechanisms. Advocates have also provided invaluable input into selected research processes. As this involvement grows, there is an increased need for training and support to increase and evaluate their effectiveness.

RAN is the advocate core for the "Center of Excellence for Individualization of Therapy for Breast Cancer" a collaborative research project with Indiana University, Hoosier Oncology Group, and sites in the United States, Canada, and Peru. The project is overseen by George Sledge Jr., M.D. and funded by the Department of Defense Breast Cancer Research Program, Office of Congressionally Directed Medical Research Programs.

Objectives of the program are to increase the advocacy community's awareness and understanding of the mission and objectives of the center and to prepare and motivate cancer survivors to inform communities about the importance of genomic research.

Methodologies: The program consisted of six webinars followed by an intensive 2-day educational program and attendance at the ASCO annual meeting. Content experts were available throughout the program. Mentors were recommended by advocate organizations or research sites and completed an application.

Webinars were 1 hour in length and were archived for later viewing. Topics for the sessions included four web lectures with *Genomics in Cancer: An Advocate's Guide* as the textbook and two sessions on *Understanding Clinical Trial Design*. The tutorials used as textbooks were both published by RAN.

The intensive educational program included both didactic and laboratory work and focused on genomics, proteomics, pharmacogenetics, clinical trial design, and proven mentoring approaches. The faculty included scientists from the center. The laboratory work included opportunities to spin down samples and follow tissue from acquisition to slide preparation and use. Participants learned about DNA, RNA, microarrays, tissue banks, mouse models, biospecimen storage, and standards for tissue banking. Experiential learning opportunities provided reinforcement and application

of the knowledge acquired. Participants attended the ASCO Annual Meeting to apply their knowledge and reported on sessions attended.

Results: The advocates currently serve as research advocates for IU/DOD, the Hoosier Oncology Group (HOG), and are reviewing grants for the Department of Defense and Susan G. Komen for the Cure Research Grants Program. They sit as community members of Institutional Review Boards; initiated legislation for an income tax check-off for funding cancer research, developed an Eagle Scout merit badge in genomics, initiated a consortium of organizations to address gaps in patient services, and serve on committees at the IU Simon tissue bank.

Conclusion: Outcomes of this pilot proved educating advocates about genomics is a successful tool to inform and motivate advocates. Participants in the program are continuing to support and communicate the importance of this new science to their constituents.

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P2-7: PLANNING GRANT FOR CoE ON OBESITY AND BREAST CANCER

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Background: This was a Planning Grant award for a Center of Excellence. Thirty-four percent of U.S. women are obese (BMI \geq 30). While obesity increases the risk of dying from breast cancer, independent of menopausal status and race/ethnicity, outcomes are worse for African Americans compared with White and Hispanic women. A woman's risk of developing breast cancer also is affected by obesity, but this relationship is complex and modified by race and the time in her life when she is obese (pregnancy, birth, childhood, premenopause, and postmenopause). Lack of a full understanding of how obesity affects both breast cancer risk and survival is a major obstacle to the eradication of breast cancer.

Methods: Working closely with an exceptional group of consumers, we created a multidisciplinary research program with experts from Georgetown University, the University of Southern California, Virginia Tech, Children's National Medical Center, the University of Tampere (Finland), and the University of Lund (Sweden). Reflecting input from consumers, our program was designed to have both immediate and long-term impact on breast cancer. To provide fundamentally new insights into the mechanisms by which timing of obesity affects breast cancer risk and into differing biology of breast cancers in obese and nonobese women, mechanistic studies were proposed in rodent models. A population study of Hispanic and African American women (Los Angeles County), a prospective molecular profiling study of breast cancer patients (DC) with independent validation in a subset of the EPIC study, and an intervention in a Finnish population also were proposed.

Results: The overarching question was: "What are the roles of obesity and metabolic syndrome in affecting breast cancer risk and survival?" Consumers were involved in all aspects of the planning grant with well-defined roles in each project in the full application and in the administration of the COE.

Aim 1: To study how obesity (and other metabolic syndrome components) during pregnancy or puberty affects mammary cancer risk in dams and offspring in animal models.

Aim 2: To determine whether timing of weight gain (body size at birth, puberty, and pregnancy) is associated with breast cancer risk in African American and Hispanic women.

Aim 3: To identify endocrine and molecular differences in breast cancers in obese and normal weight women (\pm metabolic syndrome) and build statistical predictors of recurrence risk.

Aim 4: To implement an exercise intervention program to reduce breast cancer risk and metabolic syndrome in perimenopausal and early postmenopausal women.

Conclusions: Achieving our research goals would apply interventions that reduce the impact of obesity/metabolic syndrome on breast cancer risk outcomes, understand how age at obesity affects breast cancer risk in African American and Hispanic women, identify those obese women at greatest risk of disease recurrence, and identify mechanisms that mediate interactions among fat deposits and breast epithelia and that also explain how the timing obesity/metabolic syndrome affects breast cancer outcomes, leading to new findings in prevention, detection, diagnosis, and treatment. The application for a full COE scored in the "excellent" range. A subsequent request to address the reviewers' comments was completed; the responses were acknowledged to be appropriate. The application for a full COE was not selected for funding.

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P2-8: BIOLOGICAL AND FUNCTIONAL IMAGING APPROACHES TO UNDERSTANDING BREAST CANCER METASTASIS, PROGRESSION, AND RESPONSE TO THERAPY

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The natural history of human breast cancer is characterized by the progressive selection and outgrowth of cells that possess increasingly aggressive properties. This process ultimately leads to the development of therapeutic resistance, metastasis, residual neoplastic disease, and tumor recurrence. Collectively, these features of tumor progression are responsible for the vast majority of breast cancer deaths. Nevertheless, while tumor progression constitutes a problem of unrivaled clinical importance, the mechanisms underlying it are largely unknown. As such, elucidating the molecular, cellular, and pathophysiological events that contribute to tumor progression is a critical priority in breast cancer research. The principal objective of this Center of Excellence is to develop new conceptual and technical approaches to understanding the mechanisms that contribute to breast cancer progression with a special emphasis on metastasis, residual neoplastic disease, recurrence, and genetic models for therapeutic resistance. To accomplish this, this Center of Excellence brings together researchers with expertise in developing and analyzing genetically engineered mouse models for breast cancer progression with researchers with expertise in structural, functional, and molecular imaging. The common goal of these two groups is to take a multidisciplinary approach to understanding and treating aggressive forms of breast cancer. Specifically, this Center of Excellence represents an integrated translational effort to image and understand breast cancer progression using positron emission tomography, multiphoton intravital confocal microscopy, magnetic resonance imaging, magnetic resonance spectroscopy, single-photon emission computed tomography, ultrasound, computed tomography, and optical imaging to analyze a novel series of genetically engineered mouse models for breast cancer that faithfully recapitulate key features of the human disease. The aims of this Center of Excellence will be achieved by applying a comprehensive set of cellular and molecular in vivo imaging approaches to genetically engineered mouse models for breast cancer metastasis, recurrence, residual neoplastic disease, and resistance to therapy that have been developed by the investigators of this consortium. Twenty-four investigators from seven institutions are actively participating in the experiments described in this proposal. In combining state-of-the-art. genetically engineered mouse models for breast cancer with genomics, pathology, informatics, cell biology, and a battery of innovative structural and functional imaging approaches, this Center of Excellence is attempting to improve our understanding of the basis for the aggressive behavior of subsets of human breast cancers and to thereby help illuminate the events that result in breast cancer mortality. Such information will ultimately enhance the development of more effective therapeutic approaches against highly aggressive forms of this disease by identifying the critical molecular targets and pathways by which breast cancers progress.

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P2-9: TOWARD A BETTER UNDERSTANDING OF THE RELATIONSHIP BETWEEN BREAST DENSITY AND BREAST CANCER RISK

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Women with breast tissue of high mammographic density have a 4- to 6-fold increased risk of developing breast cancer compared to women with fatty breasts. We report on a study that explores the biological basis of breast density on two fronts: (i) using acoustic properties of breast tissue to quantitate breast density safely and non-invasively and (ii) assessing the role of proteases in collagen turnover in mammary epithelial cells to gain greater insight into the cell-level processes linked to breast density. The goals of this study are to better understand the relationship between breast density and breast cancer risk and to explore the feasibility of clinical interventions for reducing that risk.

It has been demonstrated that areas of high mammographic density contain greater amounts of total collagen. As a result, we analyzed proteases implicated in collagen turnover in mammary epithelial cells (HMEC) isolated from a sample of patient biopsies of either high mammographic density (HMD-HMEC) or low mammographic density (LMD-HMEC). When grown on rBM for 48 hr in a live cell assay for degradation of dye quenched (DQ)-collagen IV, LMD-HMEC exhibited enhanced collagen IV degradation, suggesting they might differ in expression of gelatinases. However, further gelatin zymography did not reveal differences in MMP-2 and -9 expression. Since cysteine cathepsins degrade collagens endocytosed by the uPARAP pathway, we performed the DQ-collagen IV assay in the presence of an activity-based probe for cysteine cathepsins. This assay revealed co-localization of active cysteine cathepsins and degraded DQ-collagen IV. Furthermore, LMD-HMEC expressed higher levels of the cysteine cathepsins B and L than did HMD-HMEC. Our results

are consistent with the cysteine cathepsins participating in degradation of collagen IV by LMD- and HMD-HMECs, perhaps contributing to the low mammographic density associated with the LMD-HMEC.

The former work sheds light on the biological processes involved in governing tissue density. However, significant challenges also exist in the noninvasive assessment of breast density. The current standard of care relies on mammography, which clearly cannot provide accurate volumetric breast representation. Through the use of a whole-breast ultrasound tomography, ~100 patients were imaged, and two techniques were implemented for breast density evaluation. First, whole-breast acoustic velocity was determined by creating image stacks and evaluating the sound speed frequency distribution. Then, ultrasound percent density (USPD) was found by segmenting high sound speed areas from each tomogram using a k-means clustering routine. Increased sound speed was found with both increased BI-RADS Category and quantitative mammographic percent density (MPD). Furthermore, strong positive associations between USPD, BI-RADS Category, and calculated MPD were observed. These results confirm that utilizing sound speed, both for whole-breast evaluation and local segmentation, can be implemented to evaluate breast density.

While the two sides of the study presented here range from biological to physical, used in concert, they can potentially identify women who are at increased breast cancer risk and, in the long term, lead to the development of chemoprevention techniques that may mitigate that risk.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0693; Susan G. Komen for the Cure; and Michigan Economic Development Corporation.

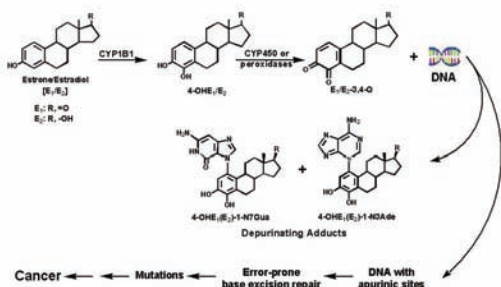
P2-10: BIOMARKERS OF BREAST CANCER RISK AND ITS PREVENTION

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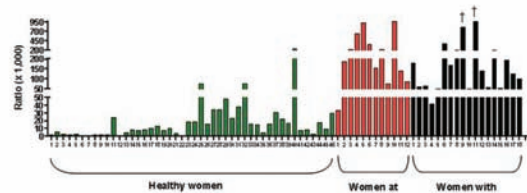
Estradiol (E_2) and estrone (E_1) can be metabolized to catechol estrogens, which can be oxidized to quinones, in particular $E_1(E_2)$ -3,4-Q. These quinones can react with DNA to form the depurinating 4-OHE₁(E₂)-1-N3Ade and 4-OHE₁(E₂)-1-N7Gua adducts. The apurinic sites generated by their depurination can induce mutations leading to breast cancer initiation.

When this occurs in humans, the N3Ade and N7Gua adducts are shed from breast tissue and excreted in urine. The estrogen metabolites, conjugates and depurinating DNA adducts in 2-ml aliquots of urine from 46 healthy women, 12 women at high risk for breast cancer (Gail model score >1.66%) and 18 women with breast cancer were purified by solid-phase extraction and analyzed by ultraperformance liquid chromatography/tandem mass spectrometry. The levels of the ratio of depurinating estrogen-DNA adducts to their respective estrogen metabolites and conjugates were significantly higher in high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$) than in control subjects. These results have been confirmed in urine and serum samples from a second set of control, high-risk, and breast cancer groups.



Major metabolic pathway in cancer initiation by estrogens

Furthermore, ingestion of 600 mg of *N*-acetylcysteine daily for 1 month by a few healthy subjects reduced the levels of estrogen-DNA adducts in urine samples. The decrease in adducts was accompanied by increased methoxy catechol estrogens, which represent the most important protective pathway in estrogen metabolism. These results support the hypothesis that formation of estrogen-DNA adducts is a critical step in breast cancer initiation (*BBA-Reviews in Cancer* 1766:63, 2006). Inhibiting adduct formation may prevent breast cancer initiation. Thus, the depurinating estrogen-DNA adducts are potential biomarkers for early detection of breast cancer risk and for use in chemoprevention.



Depurinating estrogen-DNA adducts in the urine of healthy, high risk and breast cancer women.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0229.

P2-11: ABSTRACT WITHDRAWN

P2-12: MOLECULAR PROFILING OF TISSUE TRANSCRIPTS FROM INVASIVE BREAST CANCERS AND NORMAL CONTROLS FROM BREAST-REDUCTION SURGERIES COULD YIELD NOVEL BREAST MARKERS

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In ovarian cancer, transcript analysis has been successfully employed to discover potential marker genes, including such for early detection. This was achieved by comparing the transcriptional profiles of invasive cancer tissues and normal ones obtained from prophylactic oophorectomies.

In breast cancer, marker research has focused mainly on the differences between cancer subtypes and few publications have used normal control tissues. These controls were often not well characterized or came from patients with cancer. There is mounting evidence that normal tissue from a breast with invasive malignancy may be molecularly predisposed to cancer. Such tissue may therefore not be suitable as control. We therefore have started collection of normal breast tissue from reduction mammoplasties that will be used as controls for invasive breast cancer tissues collected under the COE.

The last decade has led to a better understanding of the molecular processes underlying breast cancer. From these data we have created a database of publicly available gene and protein expression from breast and other cancers, including expression in normal tissues. This database has been mined for genes and proteins with high expression in breast carcinomas and low expression in normal tissues. Due to the scarcity of truly normal breast expression information, the latter was based mainly on other normal tissue, such as liver, ovary, and kidney.

Mining has resulted in a list of more than 100 genes with breast cancer marker potential for which PCR primers have been generated and tested. RNA has been extracted from over 200 breast tissues (invasive cancers, in-situ tumors, normal tissue accompanying the cancer, and true normal controls). PCRs will be run over the coming months. Analysis will include hierarchical unsupervised and supervised clustering. Genes with higher expression in breast tumors than controls will be subjected to protein-based expression analysis in both tissue (IHC) and serum (ELISA, Luminex). This will be initially done using readily available commercial and lab-generated antibodies. All tissues have matching sera that will be used for confirmation and validation. We expect the PCR phase to be completed by June.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0691.

P2-13: MAKING AN IMPACT ON METASTATIC BREAST CANCER THROUGH THE RAPID AUTOPSY PROGRAM—HOW TO HELP YOUR PATIENT WITH METASTATIC DISEASE MAKE A DIFFERENCE FOR THE NEXT GENERATION

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Tremendous strides have been made in the last decade to improve survival of patients with breast cancer. Even so, 15% of women diagnosed with breast cancer die. Each loss is painful for her, her family, and her providers.

Women with breast cancer are among the most educated about their disease and participate readily in clinical trials. Breast cancer patients at Johns Hopkins are participating in a unique study initiated through the DOD's Center of Excellence grant, known as the Rapid Autopsy Program (RAP). From its inception several survivors served as advocates. Questions important to the patient and the family were addressed: (1) At what point in time should we discuss with family considering having their loved one undergo an autopsy to harvest portions of organs where the disease has spread. The consensus was the patient should be asked when she is still able to make the decision for herself. The decision was made. Ask the patient. (2) Who is best qualified to ask the patient? The medical oncologist who has been taking care of the patient for some time and knows her beliefs and mindset will embark on such discussions. (3) Who will obtain informed consent and describe the study? The pathologist who would be the primary individual performing this autopsy will meet with the patient and her husband/family member while she is alert and able to ask questions. Assurance of least disruption to any funeral plans and no negative impact religious beliefs and wishes is provided. The steps of the RAP are described. The RAP team is assembled by the pathologist soon after she has passed, do the procedure, and transport her to the funeral home within hours of her death, no matter where that may be—at the hospital, home, or a hospice facility.

In the three years since its inception, the role of the survivor advocate resource of the COE has been multifaceted. For the resident RN (LS) who is also a survivor, it has been that of talking to the survivors volunteers and advocates about the program, and in one instance being personally involved in the recruitment. An in-depth research analysis of our rapid autopsy program will be undertaken, examining and measuring why patients enroll or why they don't, how participation in the program by the deceased affects members of the patients' family, what kind of feedback was expected by family members regarding research undertaken on the tissues collected through this program, in addition to whether this program is advancing our understanding the biology of metastatic breast cancer. The advocates will undertake this study in conjunction with our rapid autopsy core members.

There is no doubt that the RAP is benefiting research in metastatic breast cancer and will continue to benefit generations of researchers. Previously unknown sites of metastasis are being discovered. Multiple laboratories are pursuing questions of how primary tumors that ultimately gave rise to these metastases are different from those that did not metastasize in the same time period.

This article is dedicated to the women and their families who have participated in the RAP study and the pathology and research team who now has personal experience of being in the lives of these women during the most vulnerable time of their lives.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0595.

P2-14: A GENETIC, MOLECULAR, AND STRUCTURAL ANALYSIS OF HORMONAL CARCINOGENESIS

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Background: Hormones, especially estrogens and progestins, are known to play an important role in the development of most breast cancers. However, differences in hormone-related risk factors account for only part of the known breast cancer risk. Although mutations in a small number of genes, such as BRCA1 and BRCA2, are directly related to breast cancer development in families with hereditary breast cancer, considerable evidence indicates that genetic factors are also important in sporadic breast cancers. Breast cancer appears to be a hormonally mediated disease strongly influenced by genetic factors. This Breast Cancer Center of Excellence (BCCOE) research group is conducting a high-throughput examination of the mediators of hormonal carcinogenesis, including the estrogen and progesterone receptors and associated protein complexes, combining genetic screening of inherited polymorphisms with structural characterization of the protein products in a multi-ethnic population.

Hypotheses: The overarching problem addressed in this BCCOE is that genetic variation in mediators of hormone action in the breast contribute substantially to breast cancer risk. An improved molecular understanding of the effect of genetic variability on hormonal action in the breast will lead to the development of new therapeutic agents targeting specific genetic variants and permit implementation of new breast cancer prevention strategies.

Specific Aims: The specific aims of this program are to (1) perform a systematic screen of the genes encoding the steroid hormone receptors as well as associated protein complexes to identify potentially important polymorphisms, study the relationship

between these variants and breast cancer risk in the Multiethnic Cohort (MEC); (2) determine the three-dimensional structures of important ER- α , ER- β , and PR regulatory domains in association with proteins implicated in hormonal responsiveness and breast cancer including genetic variants of these genes; and (3) use functional assays to evaluate the significance of the structural and genetic data on receptor/cofactor activity and identify novel receptor-associated complexes.

Study Design: The research aims will be accomplished using molecular genetic, functional, and structural methods. High-throughput genotyping and sequencing technologies are used to analyze the coding, splice site, 5' and 3' UTRs, and two kilobases of the putative regulatory regions of 22 candidate genes to look for potentially relevant polymorphisms in blood from women in the Multiethnic Cohort. The three-dimensional structures of genetic variants identified in the epidemiology study population will be evaluated using protein purification and association assays, x-ray crystallography, and bioinformatics. Changes in functional activity in steroid hormone receptors and their cofactors with genetic variations are assessed using a combination of protein association assays, immunoprecipitation assays, GST pull-down experiments, transient transfection assays, and chromatin immunoprecipitation assays. This multidisciplinary approach is permitting a group of established investigators and breast cancer advocates to focus their collective attention on a single, pivotal, overarching problem in breast cancer that is at the interface of several subdisciplines.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0791.

P2-15: CELL CYCLE AND APOPTOSIS REGULATOR 1 (CCAR1), A COACTIVATOR FOR ESTROGEN RECEPTOR α , IS REQUIRED FOR ESTROGEN-DEPENDENT GROWTH OF BREAST CANCER CELLS

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University of Southern California Norris Comprehensive Cancer Center

Background and Objectives: DNA-bound nuclear receptors recruit many coactivator proteins to remodel chromatin and activate transcription. We identified a new coactivator and investigated its role in the function of estrogen receptor α (ER α) and estrogen-dependent growth of breast cancer cells. We also investigated the mechanism by which it enhances ER α function.

Methodology: Coactivator activity was investigated with transient transfection assays. Endogenous coactivator function was examined by using siRNA to reduce the endogenous coactivator level and measuring hormone-induced levels of mRNAs by quantitative polymerase chain reaction. Localization of proteins on the promoter of the pS2 gene (an endogenous target gene of ER α) in MCF-7 cells was accomplished by chromatin immunoprecipitation assays.

Results: Here we report that the protein called cell cycle and apoptosis regulator 1 (CCAR1) binds to and functions as a coactivator for ER α and several other nuclear receptors. Over-expression of CCAR1, either alone or in combination with other coactivators, enhanced the hormone-dependent and hormone-independent activity of ER α in transient reporter gene assays. In MCF-7 breast cancer cells, endogenous CCAR1 localized to the promoter of the endogenous pS2 gene in a hormone-dependent manner. Furthermore, reduction of endogenous CCAR1 levels reduced the induction of pS2 gene expression by estradiol and inhibited the estrogen-dependent growth of MCF-7 cells. The mechanism of CCAR1 coactivator function was investigated by examining the recruitment of ER, various coactivators, and RNA polymerase II to the pS2 promoter in cells with reduced levels of CCAR1. Normal hormone-dependent recruitment of ER, p160 coactivator AIB1, and coactivator CoCoA were observed when CCAR1 levels were reduced. In contrast, hormone-dependent recruitment of RNA polymerase II and several components of the Mediator complex (an important coactivator complex involved in recruitment of RNA polymerase II) was substantially inhibited in cells with reduced CCAR1 levels. CCAR1 binds to several Mediator subunits as well as components of the p160 coactivator complex.

Conclusions: We therefore propose that CCAR1 coordinates the activities of two critical coactivator complexes: the p160 coactivator complex, which is recruited directly by the DNA-bound ER; and the Mediator complex, which requires CCAR1 for its recruitment to the promoter. The requirement of CCAR1 for hormone-dependent growth of breast cancer cells suggests that CCAR1 may be a potential target for breast cancer therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0791.

P2-16: GENETIC VARIATION WITHIN THE CODING REGION OF STEROID HORMONE RECEPTOR CO-ACTIVATOR AND CO-REPRESSOR GENES DOES NOT ALTER BREAST CANCER RISK

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Background: Inherited polymorphisms are considered to play a role in breast cancer risk. Because of the known association between steroid hormones and breast cancer, we performed a systematic evaluation of polymorphisms in steroid hormone receptor coactivator genes to determine if any of these polymorphisms were associated with increased breast cancer risk.

Materials and Methods: We systematically screened the coding exons of steroid hormone receptor co-activator and co-repressor genes in an attempt to identify potentially functional polymorphisms that may serve as genetic markers of breast cancer risk. We targeted 17 genes suggested to influence transcriptional activation by steroid hormone receptors through direct binding to these receptors or through interactions with other co-activator/co-repressor protein complexes (EP300, NCoA1, NCoA2, NCoA3, CCND1, BRG1, BRM, CARM1, TRAP220, PRMT1, NCOR1, NCOR2, CBP, CoCoA, NM23, MPG, and FOXA1). Polymorphism discovery was performed by bi-directional sequencing in a multiethnic panel of 95 women with advanced breast cancer from the Multiethnic Cohort Study (MEC), composed of 19 of each African American, Japanese, Latino, Native Hawaiian, and White women. This panel was selected to have $\geq 85\%$ power to detect a potentially functional variant of $\sim 5\%$ frequency (2 of 38 chromosomes) in any one population or an overall frequency of $\sim 1\%$ (2 of 190 chromosomes). Exons containing non-synonymous variants that were observed in a single individual (i.e., singlets) were resequenced in an additional 95 cases of that racial-ethnic population. Validated non-synonymous SNPs (observed in ≥ 1 individual) were tested for association with breast cancer risk in a nested breast cancer case-control among the five racial-ethnic populations in the MEC (1,615 invasive cases and 1,962 controls).

Results: To date, we have screened 298 (86%) of 347 coding exons (sequencing of TRAP220 and CBP are in progress) and have catalogued 59 synonymous (4 per gene on average) and 37 non-synonymous SNPs (2.5 per gene on average); 22 non-synonymous variants are novel and have not been reported in dbSNP. Thus far, we have found limited evidence that common coding variation in these genes alters susceptibility to breast cancer. Modest associations were observed in some ethnic groups, but no variant was significantly associated with risk in any population or in pooled analyses (P -values > 0.05). Associations by ER/PR status and stage of disease are being investigated, as are analyses of these variants in association with endometrial cancer risk in the MEC.

Conclusion: None of the steroid hormone receptor co-activator or co-repressor genes analyzed to date have shown a significant association with increased breast cancer risk.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0791.

P2-17: GENOMIC EVOLUTION OF ENDOCRINE-RESISTANT BREAST CANCER CELL LINES REVEALS MOLECULAR ABERRATIONS CONSISTENT WITH BIOLOGICAL PHENOTYPE

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¹Translational Genomics Research Institute (TGen) and ²Fox Chase Cancer Center

Suppression of estrogen synthesis using aromatase inhibitors is highly effective in the treatment of postmenopausal women with estrogen-receptor alpha (ERalpha)-positive breast cancer. Third-generation aromatase inhibitors are superior to adjuvant tamoxifen resulting in improved disease-free survival and a lower incidence of side effects. Unfortunately, one of the consequences of long-term estrogen deprivation or exhaustive endocrine therapy is the development of drug resistance. The Jordan laboratory and others have shown that the acquisition of resistance to long-term estrogen deprivation or to selective estrogen receptor modulators (SERMs) in breast cancer cells is accompanied by an increase in malignant cell behavior. We have conducted an array-based genomic study to elucidate molecular mechanisms associated with development of endocrine resistance.

MCF-7:5C and MCF-7:2A are two ERalpha-positive human breast cancer cell lines derived from long-term estrogen deprivation of wild-type, hormone-dependent MCF-7 cells. MCF-7:TAM2 and MCF-7:RAL2 are also ERalpha-positive derivatives of MCF-7 that are resistant to the SERMs tamoxifen and raloxifene, respectively.

Whole genome expression and array-based comparative genomic hybridization (aCGH) analysis were performed on each endocrine-resistant cell line compared to the

parental MCF-7 cells. Unsupervised hierarchical clustering of global gene expression changes revealed a complex pattern of overlapping and distinct transcriptional changes. Analysis of aCGH profiles indicated both common and unique chromosomal breaks, as well as shared and unique regions of DNA gain and loss. Thus, the biological divergence of each cell line was apparent by aCGH and gene expression profiling. This suggests that long-term selective pressure exerted on MCF-7 cells results in a significant degree of genomic evolution, which contributes to observed patterns of gene expression, and presumably, biological behavior. Interestingly, amplification of the ERalpha gene (ESR1) was associated with resistance to long-term estrogen deprivation but not resistance to SERMs. Moreover, we observed several repeated genomic and transcriptional aberrations associated with long-term estrogen deprivation. For example, chromosomal regions harboring ESR1, BRCA1, and CDK4 genes are all amplified and overexpressed in MCF-7:5C and MCF-7:2A cells. Preliminary gene ontology analysis of genes differentially expressed by both MCF-7:5C and MCF-7:2A highlighted deregulated AKT signaling and cell cycle control. Elevated phospho-AKT was subsequently validated by western blot analysis. Our preliminary analysis suggests that biological drivers of endocrine resistance in each cell line model can be identified using bioinformatic approaches. We are currently prioritizing the likely molecular drivers of endocrine-resistant cell line by comparison and extraction from both genomic and gene expression data.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0590.

P2-18: COMPARATIVE GENE EXPRESSION PROFILING TO IDENTIFY UNIFYING AND SELECTIVE PATHWAYS INVOLVED IN TAMOXIFEN, RALOXIFENE, AND AROMATASE INHIBITOR-RESISTANT BREAST CANCER XENOGRAFT TUMORS

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We have developed multiple xenograft breast cancer models of antihormone resistance to the selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RAL), and to estrogen deprivation as a surrogate for aromatase inhibitors (AIs). Using these models, we have defined Phase 1 and Phase 2 antihormonal resistance based on their growth responsiveness to 17β -estradiol (E_2). Phase 1 SERM-resistant (i.e., MCF-7/RAL1) tumors are growth stimulated in response to either SERMs or E_2 , whereas Phase 2 SERM (MCF-7/RAL2 and MCF-7/TAM2) and AI-resistant (MCF-7/5C) tumors paradoxically undergo E_2 -induced regression due to apoptosis. In the current study, we compared gene expression profiles across these antihormone-resistant breast cancer models to identify unifying and selective pathways involved in their etiology and to identify genes involved in this newly discovered mode of apoptotic action of E_2 . Gene expression profiling was conducted using both Agilent 22k Human 1A (V2) Oligo Microarrays and Affymetrix Human U133 Plus 2.0 Arrays. Each tumor model showed distinct patterns of gene expression; however, hierarchical clustering showed that the Phase 2 tumors grouped together, validating our phenotypic classification. Differentially expressed genes were filtered for those genes that were commonly deregulated in both Phase 1 and 2 resistant tumors compared to wild-type MCF-7/ E_2 tumors, for those genes selectively associated with Phase 2 resistance, and for those genes differentially regulated by E_2 in the Phase 2 MCF-7/5C tumors versus wild-type tumors. In both Phase 1 and 2 resistant tumor types, nuclear receptor interacting protein 1 (NRIP1, RIP140), a corepressor of estrogen receptor α (ER α), was consistently downregulated while mucin 1 (MUC1), which stabilizes and activates ER α , was consistently upregulated. Together, these changes in expression could enhance ER α activities in antihormone-resistant tumors. Other examples of genes coordinately deregulated in both Phase 1 and 2 resistant tumor types include chemokine receptor 4 (CXCR4), BCL2-associated anthanogene 1 (BAG1), immediate early response 3 (IER3), and WW domain containing oxidoreductase (WWOX). Examples of genes that were differentially regulated by E_2 in the Phase 2 MCF-7/5C tumors versus wild-type tumors include CCAAT/enhancer binding protein delta (CEBPD), SIN3 homolog B (SIN3B), and G protein-coupled receptor 30 (GPR30).

We are examining molecular pathways indicated by the gene expression changes to understand mechanisms associated with Phase 1 and Phase 2 antihormone resistance. Currently, we are investigating a potential functional relationship between GPR30 and ER α because GPR30 is a 7-transmembrane spanning protein that binds E_2 and can mediate rapid E_2 -induced nongenomic signaling events.

E_2 -induced apoptosis in antihormone-resistant breast cancer has not yet been widely recognized but could be exploited by developing a novel treatment based on short-term, low-dose estrogen for patients who fail exhaustive endocrine therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0590.

P2-19: THE EVOLUTION OF DRUG RESISTANCE TO ANTI-HORMONAL THERAPY EXPOSES A VULNERABILITY IN BREAST CANCER

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The ubiquitous application of selective estrogen receptor modulators (SERMs) and aromatase inhibitors for the treatment and prevention of breast cancer has created a significant advance in patient care. However, the consequences of prolonged treatment with antihormonal therapy is the development of drug resistance. Nevertheless, the systematic description model of drug resistance to SERMs and aromatase inhibitors has resulted in the discovery of a vulnerability in tumor homeostasis that can be exploited to improve patient care. Laboratory studies of exhaustive antihormonal therapy demonstrate that there are at least two phases of resistance to SERMs (tamoxifen and raloxifene) and to estrogen withdrawal (aromatase inhibitors). In Phase 1 drug resistance, estrogen or a SERM promote tumor growth, but in Phase 2 drug resistance, estrogen induces apoptosis. Understanding of the new biology of estrogen action has clinical relevance. It is clear that drug resistance to antihormones evolves so that eventually the cells change to create novel signal transduction pathways for enhanced estrogen (GPR30 plus ER) sensitivity, a reduction in progesterone receptor production, and an increased metastatic potential. We have initiated a major collaborative program of genomics and proteomics to use our laboratory models to map the mechanisms of subcellular survival and apoptosis in breast cancer. The laboratory program is integrated with a clinical program that seeks to determine a minimum dose of estrogen necessary to create objective responses in patients who have succeeded and failed two consecutive antihormonal therapies. Once our program is complete, new knowledge will be available to translate the clinical care for the long-term maintenance for patients on antihormonal therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0590.

P2-20: SINGLE-ARM PHASE 2 STUDY OF PHARMACOLOGIC DOSE ESTROGEN IN POSTMENOPAUSAL WOMEN WITH HORMONE RECEPTOR-POSITIVE METASTATIC BREAST CANCER AFTER FAILURE OF SEQUENTIAL ENDOCRINE THERAPIES

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Breast cancer continues to be the most common malignancy affecting women. Although great strides have been made in the treatment and cure of early stage breast cancer, metastatic breast cancer remains incurable resulting in 40,000 deaths per year in the United States. Approximately two-thirds of all breast cancers contain the estrogen receptor (ER) and/or progesterone receptor (PgR) and are termed hormonally sensitive disease. A significant proportion of these hormonally sensitive breast cancers are dependent upon estrogenic stimulation for survival and growth. Historically, various techniques employing estrogen deprivation have been utilized to treat hormonally sensitive breast cancer; however, some of these tumors will ultimately become resistant to anti-estrogen treatment. The mechanism(s) of anti-estrogen resistance in initially estrogen-responsive tumors has not been elucidated. Preclinical data suggest that estrogen-sensitive breast cancers exposed to long-term estrogen deprivation as a result of antiestrogen treatment such as tamoxifen will evolve to no longer be responsive to such treatment and then paradoxically become stimulated to regrow during treatment. These long-term estrogen-deprived tumors exhibit increased levels of apoptosis and may be hypersensitive to the effects of estrogen in this particular setting.

To further explore the mechanisms of in vivo anti-estrogen resistance, a single-arm Phase 2 clinical trial will be performed to evaluate the clinical response rate to pharmacologic dose estrogen (Estrace) treatment in postmenopausal estrogen receptor-positive patients with metastatic breast cancer. Eligible patients will have been previously treated successfully with anti-estrogen therapy and progressed after achieving initial clinical benefit before progressing on at least two such regimens. We hypothesize that these long-term estrogen-deprived tumors will be hypersensitive to the effects of Estrace, and this will translate into clinical response, resulting in the ability to respond to further endocrine treatment, an aromatase inhibitor, in this heavily endocrine pretreated population. Our overall goal will be to evaluate the response rate to Estrace as well as the expected progression free survival with further endocrine treatment (an aromatase inhibitor) with the future plan of de-escalating the Estrace dose, thereby minimizing toxicities in this particularly targeted population.

During the first year of funding, we have focused on building the clinical infrastructure for the conduct of this multi-institutional clinical trial with Fox Chase Cancer Center (FCCC) serving as the functional "central operations center" for the adverse event monitoring, regulatory surveillance and control, and quality assurance. In collaboration with FCCC Biostatistics department, we have developed an electronic

database for the clinical information acquisition including patient enrollment logs, demographics, health history, physical exams, prior treatment(s), concomitant medications, drug compliance, adverse events/toxicities, clinical responses, clinical laboratories, and quality of life assessments. We have also sought and successfully secured funding for this investigator-initiated clinical trial as a nonrestricted grant from Astra-Zeneca Pharmaceuticals to support the clinical trial operations. Together with Johns Hopkins University Kimmel Cancer Center, we anticipate enrollment beginning early 2008.

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P2-21: SEQUENCE ALTERATIONS IN THE NUCLEAR RECEPTOR CO-ACTIVATOR 6 (NCoA6) GENE IN BREAST CANCER

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Background: Steroid receptors, members of the nuclear receptor superfamily play an important role in the initiation, promotion, and progression of breast cancer. Steroid receptor coactivators are proteins that interact with the receptor protein and other proteins, participating in the formation of the receptor coactivator complex. One such coactivator family is referred to as the p160 family and includes steroid receptor coactivator 1 (SRC1), glucocorticoid receptor interacting protein 1 (GRIP1/SRC2), and amplified in breast cancer 1 (AIB1/SRC3). These coactivators promote histone modification in order to facilitate transcriptional activation of hormone-regulated genes. NCoA6 also known as AIB3, amplified in breast cancer 3, is a nuclear receptor coactivator related to the AIB1 coactivator and is located close to AIB1 on chromosome 20. Both AIB1 and NCoA6/AIB3 are known to be amplified and overexpressed in breast cancer. Although single nucleotide polymorphisms (SNPs) are well characterized in most of these coactivator genes, little is known about somatic mutations of the steroid hormone receptor coactivators.

Materials and Methods: Genomic DNA was isolated from 11 breast cancer cell lines, one normal immortalized breast epithelial cell line, and 82 breast cancer specimens. PCR amplification of the DNA and fluorescent sequencing (Applied Biosystems) of all exons were performed for the SRC1, GRIP1, AIB1, and NCoA6/AIB3 genes in the cell line DNA. AIB3 was also evaluated in DNA from the breast cancer specimens. These tumor samples were collected from patients of four ethnicities: African-American, Latina, Asian, and Caucasian.

Results: Genetic alterations were characterized in the p160 family of proteins (SRC1, GRIP1, AIB1) in cell line DNA. A total of seven known SNPs and one new synonymous mutation were identified in these genes. No new non-synonymous mutations were identified in the cell line DNA, therefore no tumor DNA was sequenced for these genes. In contrast, two novel non-synonymous mutations were identified in cell line DNA for NCoA6/AIB3, one each in SKBR3 and MDA-MB-435. Similarly, evaluation of AIB3 in 82 breast cancer specimens demonstrated four additional non-synonymous mutations. Each one of these mutations was identified in one breast tumor. In addition to the six new non-synonymous mutations presented above in AIB3, there were six new silent mutations and four known SNPs.

Conclusion: Six new NCoA6/AIB3 non-synonymous sequence alterations were identified within the C-terminal region of the gene or close to it. The C-terminal domain, containing four of these mutations, is known to interact with DNA dependent protein kinase as well as other coactivator proteins. Therefore, sequence modifications in this region may alter the function of AIB3.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0791.

P2-22: GENE EXPRESSION PROFILING, LOSS-OF-HETEROZYGOSITY, COMPARATIVE GENOMIC HYBRIDIZATION, AND GENE METHYLATION ISLAND ANALYSIS OF AN INTERNATIONAL COHORT OF WOMEN WITH LOCALLY ADVANCED BREAST CANCER

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Background and Discussion: Locally advanced breast cancer (LABC) is an advanced (Stage III) presentation of breast cancer, classically a tumor 5 cm or larger, but generally without distant metastasis at the time of presentation. Most LABC will progress to metastatic disease in several years following presentation if untreated. LABC is the face of breast cancer worldwide, accounting for roughly half of all breast cancers presenting clinically, yet it is an understudied disease. In the United States, LABC accounts for roughly 15% of breast cancer overall, but up to 30% in medically underserved immigrant and minority women. A significant reason for the lack of

understanding of LABC is the preponderance of barriers to joining clinical trials for medically underserved women and therefore the availability of patient tumor tissues obtained in a consented manner useful for scientific studies. Through our CDMRP funded Center of Excellence for LABC, we are seeking to understand the genetic basis of LABC so as to inform treatment decisions. We are characterizing the genetics of LABC by building an international clinical trial that includes medically underserved women at Bellevue Hospital Center in New York City, one of the largest public hospitals in the U.S., in Tygerberg Hospital of Stellenbosch University in South Africa, Centro Medico Oncology Hospital in Mexico City, and the Amrita Institute of Medical Sciences, Cochin, India. Through these opportunities we are prospectively collecting a multi-ethnic LABC tissue repository of all patient tissues, both pretreatment and post-treatment, in paraffin, as frozen core biopsies and blood specimens for women treated for LABC through a common protocol (paclitaxel and concurrent radiation). This cohort of tumor tissue is presently being used to determine gene expression profiles (35 patients to date will be reported on). We have also engineered from paraffin specimens the ability to conduct single nucleotide polymorphism (SNP) studies using Affymetrix 500,000 SNP chips, as well as determination of heterozygosity (LOH) and Comparative Genomic Hybridization (CGH) to determine gene copy number alterations, both inherited and sporadic that might be associated with presentation of LABC and/or treatment response. Using a proprietary chip hybridization format known as ROMA, both a high density CGH map and the identification of gene methylation islands that influence gene expression are being conducted, and to date include the same 35 patient tumor specimens. These data will be reported as well.

Conclusions: The results of these studies will compare women with LABC from different race and ethnicity cohorts. These studies will characterize the genetic alterations associated with LABC, with the clinical presentation of initially non-metastatic breast cancer, and polymorphic differences in incidence and presentation.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0905 and Breast Cancer Research Foundation.

P2-23: A TRANSLATIONALLY CONTROLLED ANGIOGENIC SWITCH IN LOCALLY ADVANCED BREAST CANCER

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Background: Locally advanced breast cancers (LABC) form large late-stage tumors generally without distant metastases at the time of clinical presentation. Tumor progression requires the development of a tumor vasculature, generally induced in response to tumor hypoxia through a process known as an angiogenic switch. This process achieves elevated expression of VEGF and other pro-angiogenic factors. Translational control plays a key role in cancer development and progression, impacting on tumor cell proliferation, response to hypoxia and the ability to promote angiogenesis. We and others have shown previously that during tumor hypoxia, the activity of the kinase mTOR is reduced, the translation regulator 4E-BP1 is activated and suppresses translation of most (capped) mRNAs, while promoting translation of mRNAs that orchestrate angiogenesis (VEGF-A, FGF), the hypoxia response (HIF1a) and prevent cell death (Bcl2), among others. We show that the majority of LABC tumors overexpress 4E-BP1, which promotes a striking 2-3 fold increase in tumor growth and angiogenesis through translational overexpression of VEGF, Bcl2, and HIF1a mRNAs under hypoxia. Increased expression of these genes is shown to occur through a novel angiogenic switch that functions at the level of translational control and is critical for the development of these large, highly angiogenic tumors.

Methods: Studies used pretreatment tumor biopsies from LABC and other breast cancers, studied by immunohistochemistry. Breast cancer cell lines were engineered to express 4E-BP1 or silence genes using lentivirus transformation. VEGF levels were determined by ELISA. Angiogenesis was investigated in the 10-day chorioallantoic membrane of the fertilized chick ovum and in mouse models. Tumorigenesis studies involved xenotransplant models in nude mice.

Results and Discussion: Strong overexpression of 4E-BP1 and translation initiation factor eIF4G1, were found specifically in almost 80% of LABC (T2, T3) tumors compared to low-level expression in most earlier stages of disease. We developed breast cancer cell lines to emulate LABC and showed that similar overexpression of 4E-BP1 and eIF4G1 orchestrate a hypoxia-controlled angiogenic switch in translational control that drives tumor vascularization and growth. Using model animal systems and interfering RNA studies, elevated 4E-BP1 and eIF4G promoted inhibition of cap-dependent mRNA translation under hypoxia and promoted selective translation of mRNAs containing internal ribosome entry sites (IRES), such as VEGF, Bcl2 and HIF1a, more than doubling tumor growth and angiogenesis. Remarkably, this setting suppressed tumor metastasis through a mechanism not yet understood. Our studies have identified genes that are vital for the development of these large advanced and highly angiogenic tumors. This work provides a new understanding of LABC, and it identifies a new strategy and window of opportunity for the treatment of this disease.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0905; Breast Cancer Research Foundation; and American Cancer Society.

P2-24: BIOMARKERS OF METASTATIC BREAST CANCER-MULTIMODAL ANALYSIS AND IDENTIFICATION OF DRUGS TARGETED TO OVEREXPRESSED HOXB7

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Great strides have been taken in recent decades in more effective treatment of breast cancer. However, achieving a permanent cure by eradicating metastatic breast cancer has remained elusive. As a first step to devising suitable therapies, identification of alterations in expression levels of genes/proteins that are typical of metastatic breast cancer and its distant metastasis is necessary. It is also clear that communication between tumor cells and other cells in the microenvironment, such as the vascular endothelial cells (ECs), the stromal fibroblasts, and immune cells, is critical for tumor growth. We have performed SAGE and microarray analysis to identify several epithelial molecular markers overexpressed in primary breast cancers. Considerable progress has been made in understanding the biology and function of a transcription factor, HOXB7, in breast development and cancer. Biological and functional analysis of HOXB7 was performed at the biochemical and molecular level, and using multiple imaging modalities.

HOXB7 is overexpressed in approximately 30% of primary breast cancers and to greater extent in bone metastases. By ¹H NMR imaging studies, altered choline phospholipids and increased vascular permeability were seen in HOXB7-expressing MCF-7 cells. MCF-7-HOXB7 cells express increased levels of tyrosine kinase receptors (e.g., EGFR and HER-2) as well as increased levels of ERα and appeared to be similar in this regard to MCF-7 cells that are resistant to letrozole, a model of endocrine-resistant breast cancer. A search for compounds that target HOXB7 and inhibit cell growth in vitro and also tumor growth in vivo revealed that HOXB7-overexpressing MCF7 cells respond to nM levels of VN/14-1, a newly synthesized atypical RAMBA (retinoic acid metabolism blocking agent)/aromatase inhibitor active against both letrozole- and tamoxifen-resistant breast cancer cells. Consistent with this finding, the aromatase inhibitors letrozole and anastrozole are effective in suppressing proliferation of MCF-7-HOXB7 cells in vitro at IC50 concentrations 1-2 nM. This suggests that patients whose breast tumors overexpress HOXB7 and are resistant to treatment with tamoxifen may be responsive to sequential treatment with the aromatase inhibitors. In addition, the tyrosine kinase inhibitor gefitinib and AEE (Novartis) inhibited MCF-7-HOXB7 cell proliferation. The m-Tor inhibitor RAD-001 (Novartis) was also an effective inhibitor of HOXB-7 cells at low nM concentrations. These results complement our findings in letrozole-resistant cells and indicate that crosstalk is occurring between estrogen signaling and tyrosine kinase receptor pathways. VN/14-1 is poised to enter clinical trials. HOXB7 confers tamoxifen resistance and metastasis; both aspects were replicated in a newly developed model of mammary tumors arising in double transgenic HOXB7/neu mice.

From this knowledge, we will finally devise combinations of immune and drug therapy that target the tumor and its microenvironment to prevent and treat metastatic breast cancer and its micro- and macrometastasis most effectively.

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P2-25: STRUCTURAL AND CULTURAL BARRIERS TO CLINICAL DIAGNOSIS OF LOCALLY ADVANCED BREAST CANCER (LABC) IN A MULTIETHNIC COHORT: FINDINGS FROM MEXICO, SOUTH AFRICA, INDIA, AND THE UNITED STATES

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The Department of Defense Center of Excellence in Locally Advanced Breast Cancer (COE-LABC) based at New York University supports a suite of interlocking research projects to assess the clinical, biological, cultural, and epidemiological characteristics of LABC in a transnational multiethnic cohort of patients. One of these projects is an in-depth investigation of sociocultural and structural factors involved in patients' presentation for LABC, a disease that claims a disproportionately high number (50%–70%) of breast cancer cases in developing countries and 20%–30% of cases in medically underserved and minority communities in the United States. At Bellevue Hospital in New York, 50% of new breast cancer patients lack health insurance, and more than 50% are recently arrived immigrants who speak Chinese or Spanish. The Breast Health Global Initiative and other efforts seek to outline economically feasible guidelines for improvement of screening, prevention, and treatment in resource-limited settings. However, there has been little attention given to the impact of cultural or

structural factors, or how such factors are unique or similar across national settings. In behavioral research on breast cancer, a central focus has been on individual level beliefs such as fatalism or denial, with little inquiry into political economic forces that enable or disable patients' behaviors or beliefs in the first place. An in-depth semi-structured interview and Illness Perception Questionnaire (IPQ) was administered to 50 newly diagnosed LABC patients at participating hospitals in Mexico, South Africa, India, and New York. Patients were asked to report when they first noticed a lump in their breast (time 1); when they first presented to a doctor (time 2); and when they received a confirmed diagnosis of LABC at the participating COE hospital (time 3).

Participants were also asked to qualitatively describe socio-cultural meanings and experiences associated with each of the 3 time points. While there was an average delay of 6.6 weeks between times 1 and 2, there was a 24-week delay between times 2 and 3, indicating a potentially strong impact of structural factors such as insufficient diagnosis and lack of referral/follow up to cancer services. In addition, participants described political economic barriers such as lack of health services in their neighborhood or township, lack of transportation, job insecurity, lack of money for health care, and gender inequality (i.e., in South Africa where participants were uncertain whether they could start treatment due to insufficient support from employers, husbands, tribal leaders). Findings indicate that doorways to access diagnosis for LABC vary across national settings. At the same time, similar patterns of delay from first presentation to confirmed diagnosis across the 4 sites indicates that primary-to-tertiary referral patterns warrant further attention in tandem with individual factors such as lack of awareness about breast lumps or breast cancer. While structural factors appeared significant across all 4 sites, participants in the developing country sites (Mexico, South Africa, India) reported additional barriers including lack of infrastructure/resources for specialty cancer care; societal attitudes about women's health as a low priority; lack of family savings for health care; and geographic gaps between cancer care centers (as far as 500 km apart).

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P2-26: PRIMARY CONCURRENT CHEMORADIATION OF LOCALLY ADVANCED BREAST CANCER: FEASIBILITY IN INDIA

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New York University School of Medicine (NYU) was awarded a Center of Excellence (COE) grant to study locally advanced breast cancer (LABC) in the United States, Mexico, South Africa, and India. Ethnic differences in incidence and outcome of LABC patients are well documented, but it is unknown whether they mainly reflect socioeconomic and cultural factors that delay detection and affect access to optimal care or whether they are also due to genetic factors affecting tumor characteristics and response to therapy. Central to the COE is the conduct of a Phase II study of concurrent paclitaxel and radiation to assess whether the tolerance and response of LABC patients in other countries are comparable to that observed in the United States (Formenti et al. 2003). The regimen consists of paclitaxel, 30 mg/m² twice per week given IV over 1 hour, starting during first week of radiotherapy to the breast, axilla, and supraclavicular area (45Gy + 14Gy to original palpable tumor). Surgery follows at least 2 weeks after completion of chemoradiation. The COE protocol recently received local and international IRB approvals and is open for accrual at each site. In preparation for accrual to the primary protocol, we reviewed patterns of toxicity and response in women accrued to a previous study in which 4 cycles of AC (doxorubicin and cyclophosphamide) were given prior to concurrent paclitaxel and radiation in 11 patients at Amrita Institute of Medical Sciences (AIMS) in India and 11 patients at NYU-Bellevue.

Results: Pathological response rates were the same (36%) at both AIMS and NYU. Acute toxicities encountered during concurrent paclitaxel and radiation and during the 4 cycles of initial AC are summarized as follows: *Dermatitis*—grade I (4 cases at NYU, 1 case at AIMS); grade II (4, 5); grade III (2, 4); grade IV (0, 1). *Pneumonitis*—grade I (0, 0); grade II (0, 1); grades III-IV (0, 0). *Hematological*—grade I (2, 4); grade II (0, 6); grade III (2, 0); grade IV (0, 0). *Nausea/vomiting*—grades I (3, 0); grade II (3, 0); grades III-IV (0, 0). *Fatigue*—grade I (7, 9); grade II (2, 0); grades III and IV (0, 0). *Diarrhea*—grade I (2, 0); grade II (1, 0); grades III-IV (0, 0). *Alopecia*—grade I (0, 0); grade II (11, 11). An increase of grades III-IV dermatitis was noted in 5/11 patients treated at AIMS versus 2/11 at NYU. Hematological toxicity, mainly grades I-II anemia, was observed in 10/11 patients in India after AC, but only in 2/11 patients at NYU. Differences of radiotherapy technique and skin care may be responsible for differences in skin toxicity. Similarly differences in supportive care may account for differences in chemotherapy-related toxicity. A site visit to India is planned in early January to assess cause for detected differences and to establish common standard of treatment and supportive care. Accrual to the new protocol of paclitaxel and radiation is deferred until after the site visit. Presumably, in the new protocol, the omission of primary AC upfront will likely reduce hematologi-

cal complications. In conclusion, the current report supports cautious translation to other health care systems and ethnic groups. While differences encountered may be ascribed to technical causes, different pharmaco- and radiation genomics among the 2 cohorts cannot be excluded at this time.

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P2-27: PROTEOMIC ANALYSIS OF PHOSPHOTYROSINE-CONTAINING PROTEIN COMPLEXES DURING ESTROGEN-INDUCED PROLIFERATION AND APOPTOSIS IN MCF-7 HUMAN BREAST CANCER CELLS

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We have used a panel of MCF-7 cells in vitro that have been E2-deprived (ED) for several years to replicate resistance to aromatase inhibitors. The parental MCF-7 cell line responds to estradiol (E2) with growth whereas E2 causes rapid apoptosis in estrogen-deprived clonal variant MCF-7:5C cells. This study aimed to identify early signaling pathways underlying the low-dose estrogen-induced apoptosis in the MCF-7:5C breast cancer cell line, which may have significant implications in designing novel therapies for anti-estrogen drug-resistant breast cancers. To identify these pathways, we have established a process to use immunoprecipitation to isolate protein signaling complexes involved in estrogen or growth factor signaling thereby fractionating the cells' proteome. An antibody recognizing tyrosine phosphorylated proteins was used to pull down unique complexes of proteins in estrogen-treated versus untreated cells followed by 1D-SDS PAGE. Bands determined to be unique for each treatment condition, in duplicate, after 2 hours treatment with E2, were excised and subjected to mass spectroscopic analysis (MS/MS). The results were analyzed using the iProXpress system, facilitating functional annotation of identified proteins, and Ingenuity[®] Systems pathway analysis software, allowing the proteins to be mapped to known cell signaling pathways. Here, we have identified 25 unique protein targets in E2-treated MCF-7 cells and 15 unique protein targets in E2-treated MCF-7:5C cells. In E2-treated MCF-7 cells, 7 proteins are associated with a signaling network with nodes centered on H-RAS and coatamer A/B, and 17 proteins are associated with a signaling network with nodes centered around TNF- α and IL-1 β , both networks of which are involved strongly in cancer. In contrast, analysis of E2-treated MCF-7:5C cells revealed that 9 proteins are associated with a signaling network with nodes centered around retinoic acid, NF- κ B, PTEN, and p38 MAPK, and 3 proteins are associated with a signaling network with nodes centered around hsp70, hsp90, and cMyc; the former network has been found in cancer signaling, and tissue development signaling whereas the latter network has been associated with endocrine system development/function and lipid metabolism. The next steps in this study are to confirm the existence of these protein complexes by immunoprecipitation and western blot analysis with specific antibodies and to determine if siRNA targeting of relevant activating pathways affects the E2-induced apoptotic response.

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P2-28: PROTEOLYTIC AND SIGNALING PATHWAYS IN BREAST CANCER: NOVEL MODEL FOR FUNCTIONAL IMAGING AND THERAPEUTIC SCREENING

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Background: The increase in mammographic screening has led to greater numbers of women being diagnosed with mammary ductal carcinoma in situ (DCIS). A major problem is that some DCIS lesions rapidly progress to invasive ductal carcinomas (IDC) whereas other DCIS lesions remain indolent, and we are not presently able to predict which is which. Key proteases are increased in expression in human breast biopsies at the point of transition from pre-invasive DCIS to invasive carcinomas. For example, the cysteine protease cathepsin V/L2 and the matrix metalloproteinase (MMP)-11 are the two proteases comprising the invasion signature of the recurrence-score model used in the Oncotype DX clinical test to direct therapeutic decisions for women with early stage breast cancer. Proteolytic pathways also are regulated through other mechanisms such as secretion and activation. We have shown that aberrant signal transduction that is driven by dysregulated activation of Ras small GTPases and kinases can lead to increased pericellular proteolysis. Clearly, the constituents of the proteolytic pathways that contribute to malignant progression need to be defined and validated before considering their potential as therapeutic targets.

Objective: We are testing the hypothesis that the transition from pre-invasive DCIS to invasive carcinomas is mediated through alterations in proteolytic pathways, both in the epithelial cells and DCIS-associated cells, and that dysregulated activation of Ras small GTPases and kinases contributes to the induction of these aberrant proteolytic pathways.

Methodology: We modeled progression of normal mammary tissue through pre-malignant lesions to carcinoma using 3D reconstituted basement membrane (rBM) overlay cultures of a progression series of cell lines derived from MCF10A (10A) breast epithelial cells grown alone and in triple layer (bottom layer of collagen I, middle layer of rBM, top overlay of 2% rBM) cocultures with breast fibroblasts plus or minus breast myoepithelial cells. To analyze effects on proteolysis, we used live cell confocal imaging. We modulated expression or function of target proteins using retroviral transduction and selective small molecule inhibitors.

Results: We have developed a 3D triple layer coculture model designed to recapitulate mammary tissue in vitro: MAME (mammary architecture and microenvironment engineering). Cell lines of the MCF10A lineage [10A; 10.DCIS; and CA1d (carcinoma)] were grown alone and in triple layer cocultures with WS-12Ti (tumor-associated human breast fibroblasts). The MAME cocultures also exhibit the progression observed in the 3D rBM overlay cultures, with the addition of fibroblasts increasing the amount of proteolysis observed and enhancing the invasiveness. Addition of breast myoepithelial cells [a kind gift of K. Polyak (Dana-Farber)] reduces the proteolysis and reverses the invasive phenotypes of the 10.DCIS and CA1d cocultures.

Conclusion: The MAME coculture model recapitulates the architecture of human breast tissue, thus serving as an in vitro tool for screening of small molecule inhibitors. Validating proteases and proteolytic pathways key to progression of pre-invasive DCIS to IDC and the pathways that regulate them should discover potential targets for therapeutic intervention as well as candidate biomarkers to identify those DCIS lesions that will rapidly progress to IDC.

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P2-29: A GLUCOSE-REGULATED PEPTIDE 78 (GRP78)-BINDING MOTIF FUSED TO THE PROAPOPTOTIC SEQUENCE D(KLAKLAK)₂ CAN INDUCE TUMOR IMMUNITY

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The impact of antigen-specific immune tolerance on responses to an HER-2-directed, GM-CSF-secreting vaccine in a murine model of HER-2⁺ (NT) breast cancer has been previously demonstrated. Specifically, a GM-CSF-secreting, HER-2-specific vaccine can induce complete tumor rejection in 100% of nontolerant tumor-bearing FVB/N mice; the same vaccine is completely ineffective in tolerant, tumor-bearing *neu*-N mice transgenic for the rat proto-oncogene *neu*. Abrogating regulatory T cells with low-dose cyclophosphamide (CY) can augment vaccine activity in tolerant *neu*-N mice, curing up to 25% of tumor-bearing animals. The immune response of cured FVB/N and *neu*-N mice is characterized by a predominance of CD8⁺ T cells specific for the rat *neu* epitope RNEU₄₂₀₋₄₂₉. We hypothesized that the proapoptotic peptide GRP78-d(KLAKLAK)₂, where glucose-regulated protein 78 (GRP78) can function as a membrane-associated molecular chaperone for tumor targeting, could also induce effective tumor immunity. We treated nontolerant parental FVB/N mice bearing 7-day NT tumors (3-4 mm) with intraperitoneal injections of a chimeric peptide composed of GRP78 binding motifs fused to the programmed cell death-inducing sequence d(KLAKLAK)₂ given three times per week. The peptide and the *neu*-targeted, GM-CSF-secreting vaccine were equally effective in inducing tumor rejection in FVB/N mice by 40 days after tumor challenge. Tumor rejection was associated with T helper type 1 response, and an IFN- γ -secreting, RNEU₄₂₀₋₄₂₉-specific CD8⁺ T cell response with both peptide and vaccine. HER-2-specific antibodies were also induced. There was no tumor rejection, T cell, or antibody response when mice were treated with vehicle alone. In contrast, the peptide alone failed to impact tumor outgrowth until 25 days after tumor challenge in *neu*-N mice bearing 3-day NT tumors when a modest delay in comparison to control (vehicle-treated) mice emerged. Abrogating regulatory T cell-mediated immunosuppression with low-dose CY given prior to peptide administration most effectively delayed tumor outgrowth in tolerant *neu*-N mice. Treatment with peptide alone or CY + peptide induced NT tumor cell-specific immune responses in *neu*-N mice. The antitumor activity of GRP78-d(KLAKLAK)₂ may be in part immune based, and this supports combining this novel pro-apoptotic therapy with tumor vaccines.

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HBCU/MI PARTNERSHIP TRAINING AWARDS I

Poster Session P3

P3-1: BREAST CANCER CELLS DOWN-REGULATE TRISTETRAPROLIN IN CO-CULTURED MACROPHAGES: A POSSIBLE MECHANISM FOR SUSTAINING INFLAMMATION AND ANGIOGENESIS IN THE TUMOR MICROENVIRONMENT

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Tumor-associated macrophages (TAMs) promote tumor progression through the secretion of inflammatory cytokines and growth factors. Control of inflammatory cytokine gene expression occurs at several levels including the regulation of mRNA stability. Tristetraprolin (TTP), an evolutionarily conserved protein, binds to adenine and uracil (AU)-rich elements (AREs) in the 3'-untranslated region of several mRNAs, including its own, targeting them for degradation. TTP destabilizes mRNAs of several inflammatory cytokines, including interleukin (IL)-1, IL-2, and IL-8, and vascular endothelial growth factor (VEGF). Both IL-8 and VEGF have been implicated in angiogenesis and invasion. In response to inflammatory mediators, activation of a p38 MAPK/ MAPK-activated protein kinase 2 (MK2) axis leads to inhibition of TTP and an increase in the stability of ARE-containing mRNAs. We hypothesized that breast cancer cells would regulate TTP expression in TAMs to sustain an inflammatory and pro-angiogenic microenvironment. We used an in vitro co-culture system, reverse transcription-polymerase chain reaction, and western blotting to examine breast cancer cell control of TTP mRNA and protein levels in co-cultured macrophages. LPS activation of macrophages caused a decrease in TTP mRNA levels. Whereas MCF-7 cells did not cause a change in TTP mRNA levels in co-cultured macrophages, MDA-MB-2321 cells caused a slight decrease in TTP mRNA levels. However, MCF-7 cells caused a 36% reduction, while MDA-MB-231, a more tumorigenic and metastatic cell line, induced a 57% reduction in TTP protein levels in co-cultured macrophages. Lipopolysaccharide (LPS) activation caused a 32% increase in macrophage TTP, and neither cancer cell line reduced TTP levels in LPS-activated macrophages. The observed increase in TTP protein levels in activated macrophages is consistent with earlier reports that LPS-induced phosphorylation of TTP increases its stability while decreasing its affinity for AREs. Thus, TTP protein levels may be higher in TAMs despite a decrease in mRNA levels. Our results suggest that there may be a correlation between the aggressiveness of tumor cells and their ability to sustain an inflammatory and pro-angiogenic microenvironment via the reduction of TTP levels in stromal macrophages. These results contribute to an understanding of the mechanisms by which cancer cells alter TAM physiology to promote angiogenesis and metastasis.

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P3-2: DIABETES, PHYSICAL ACTIVITY, AND BREAST CANCER AMONG HISPANIC WOMEN

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In most studies, diabetes has been associated with increased breast cancer risk, while physical activity has been linked to reduced breast cancer risk. Hispanic women have high rates of diabetes, but fairly low rates of physical activity. In the current study, we assessed whether physical activity modified the effect of diabetes on breast cancer in Hispanic women.

We used data from a case-control study of breast cancer among Hispanic women ages 30 to 79 conducted between 2004 and 2007 on the Texas-Mexico border. In-person interviews were completed with 176 incident breast cancer cases ascertained through mammography centers, and 464 controls who had two consecutive negative screening mammograms (with respective response rates of 96% and 75%). Diabetes or borderline diabetes was based on self-report.

After adjustment for age, menopausal status and body mass index, there was a reduction in breast cancer risk associated with diabetes (odds ratio [OR] 0.74, 95% confidence interval [CI] 0.49-1.11) and with physical activity (upper quartile of metabolic equivalent OR 0.55, 95% CI 0.32-0.95). While there was no association between diabetes and breast cancer among women who did not engage in physical activity (OR 0.93, 95% CI 0.56-1.55), the association between diabetes and breast cancer was greatly reduced among women who exercised (OR 0.34, 95% CI 0.18-0.87). Stratification by menopausal status and further adjustment for alcohol intake and postmenopausal hormone use did not materially change these associations.

Although non-significant, our negative association between diabetes and breast cancer was somewhat unexpected since a recent meta-analysis reported a significant positive association (pooled relative risk 1.20) that was not modified by physical activity. Our study is one of the first studies to investigate the association between diabetes and breast cancer among Hispanic women. In addition to hormonal mechanisms, potential mechanisms that warrant exploration are related to severity and treatment of diabetes. Should larger studies confirm our results, the reduction in risk among diabetic women,

especially those that exercise, may help explain the lower rate of breast cancer among Hispanic women.

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P3-3: BODY COMPOSITION AND POSTMENOPAUSAL BREAST CANCER IN HISPANIC WOMEN

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Obesity and body fat distribution have been shown to be associated with increased risk of postmenopausal breast cancer in many ethnic groups. Most of the studies have evaluated these associations using body mass index as a surrogate marker of obesity. In the current study, we evaluated whether central adiposity and obesity assessed by both bioelectrical impedance analysis and body mass index are associated with breast cancer risk in postmenopausal Hispanic women.

We used data from a case-control study of breast cancer among Hispanic women ages 30 to 79 conducted between 2004 and 2007 on the Texas-Mexico border. In-person interviews were completed with 176 incident breast cancer cases ascertained through mammography centers, and 464 controls who had two consecutive negative screening mammograms (with respective response rates of 96% and 75%). Body mass index, waist circumference, and percent body fat are based on measured values. This analysis is restricted to postmenopausal women (123 cases, 371 controls).

After adjustment for age and postmenopausal hormone use, there was a slight non-significant increase in breast cancer risk associated with body mass index (upper quartile odds ratio [OR] 1.30, 95% confidence interval [CI] 0.67-2.51) and waist circumference (upper quartile OR 1.39, 95% CI 0.75-2.57), but no association with percent body fat (upper quartile OR 0.98, 95% CI 0.51-1.86). When we restricted the analysis to women who had undergone menopause at least 4 years prior there was a strengthening of the effect for all measures of body composition (upper quartile of body mass index OR 1.45, 95% CI 0.68-3.10; upper quartile of waist circumference OR 1.40, 95% CI 0.70-2.79; upper quartile of percent body fat OR 1.28, 95% CI 0.60-2.71). Further adjustment for physical activity did not materially change these associations.

Increased body mass index and central adiposity were positively associated with postmenopausal breast cancer risk in our study of Hispanic women. The effects of obesity and central adiposity were accentuated with longer time since menopause which may reflect the time necessary for the production of estrogens by adipose tissue to become the main source of endogenous estrogen. Should larger studies confirm our findings, this may explain the conflicting evidence for the association between measures of body composition and postmenopausal breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0274.

P3-4: REVERSIBLE INACTIVATION OF CDC25A BY ESTROGEN AND ANTIESTROGEN-INDUCED REACTIVE OXYGEN SPECIES MAY BE INVOLVED IN THE PHOSPHORYLATION OF p27

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Reactive oxygen species (ROS) reversibly regulates cysteine-based phosphatases. The CDC25s, dual-specificity tyrosine phosphatases, possess the necessary elements to function directly in redox control of the cell cycle. ROS can activate kinases ERK, and Akt that are known to indirectly, and directly phosphorylate p27, respectively. We postulate that the reversible inactivation of CDC25A by estrogen or antiestrogen-induced ROS is a potential key player in the phosphorylation of p27 by ERK or AKT. To test our postulate, our first objective was to investigate whether the effects of estrogen or antiestrogen on CDC25A and p27 are mediated via redox signaling. Previously an increase in CDC25A mRNA has been reported after 6 hrs of E2 treatment and an increased synthesis of CDC25A protein was detected from 10 to 12 hrs after E2 treatment. However, they did not examine the stability of CDC25A protein. Our initial study revealed that the level of CDC25A protein did not change immediately after E2 exposure; however, we observed an increase in the level of its protein as early as 3 hrs after E2 exposure. This time period is well before its mRNA induction starts. This led us to believe that E2 treatment appears to increase its stability. We further looked for any post-translational modification from E2-exposure because full enzymatic activity of CDC25A requires phosphorylation at multiple Ser and Thr residues in the amino-terminal of the protein. In contrast to UV or IR effects on CDC25A in cells, we observed a rapid decrease in serine phosphorylation of CDC25A in response to physiological concentrations of estrogen that paralleled the decrease in phosphatase

activity. A similar decrease in the serine phosphorylation of CDC25A and its activity was observed in response to the exposure of cells with hydrogen peroxide. We further examined whether CDC25A represents a target of estrogen-induced oxidants in the intact cells. The level of -SH residues in CDC25A was lower in 100 pg/ml E2-treated cells compared to the vehicle-treated cells. This indicates that the increased oxidation of -SH residues of CDC25A in response to E2-induced ROS. CDC25A is also phosphorylated at tyrosine residues, and in contrast to serine phosphorylation, tyrosine phosphorylation of CDC25A was enhanced in cells treated with estrogen or H2O2. Estrogen-induced ROS may act directly at CDC25A and is responsible for its inactivation, because we can prevent E2-induced inactivation of CDC25A by co-treatment with antioxidant, NAC. Tamoxifen mediates serine phosphorylation of p27 and this was inhibited by overexpression of the antioxidant enzyme MnSOD in MCF-7 cells. And MCF-7 cells exposed to the oxidant hydrogen peroxide also increased p27 serine phosphorylation as detected with an anti-phosphoserine antibody. Ebselen co-treatment appears to counteract the effects of tamoxifen on estrogen-induced expression of CDC25C. These initial results show support toward our hypothesis, with increased ROS formation by estrogen and antiestrogen treatment, attenuation of CDC25A activity by estrogen-induced ROS, and inhibition of tamoxifen-mediated p27 phosphorylation by overexpression of the antioxidant enzyme MnSOD.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0417.

P3-5: EBSELEN CO-TREATMENT COUNTERACTS THE EFFECTS OF ANTIESTROGEN ON ESTROGEN-INDUCED GROWTH OF BREAST CANCER CELLS AS WELL AS RESTORES THE GROWTH INHIBITORY EFFECTS OF ANTI-ESTROGEN IN RESISTANT CELLS

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The purpose of is to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift toward a pro-oxidant environment of cells as a result of oxidative stress. Our first objective was to demonstrate that indeed antiestrogens counteract estrogen actions by controlling ROS formation. Our study showed that tamoxifen exposure to MCF-7 increases ROS formation, based on the increased oxidation of the oxidant-detecting probe DCFH in tamoxifen-treated cells. We confirmed this finding using confocal microscopy showing that tamoxifen indeed increases intracellular levels of ROS. We observed similar results with another antiestrogen, fulvestrant. Estrogen- or antiestrogen-mediated increased ROS formation was inhibited by the antioxidant ebselen. To understand cell proliferation effects at the molecular level, we have investigated the co-treatment of antioxidant plus antiestrogen on the estrogen-induced expression of cell cycle genes. Ebselen co-treatment appears to counteract the effects of tamoxifen on estrogen-induced expression of CDC25C and PCNA at mRNA level. To test our postulate that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift toward a pro-oxidant environment of cells as a result of oxidative stress from chronic antiestrogen treatment therapy, we performed cell proliferation experiments by BrdU incorporation. Initial results for this assay on antiestrogen-resistant cells LCC2 and LY2 showed that ebselen restored inhibitory action of antiestrogen in these cells. These initial results show support toward our hypothesis, with increased ROS and decreased cell proliferation in breast cancer cells upon co-treatment with antiestrogen, expression of a cell cycle gene, and the co-treatment with ebselen appearing to restore growth inhibitory effects of antiestrogen in resistant cells. As the overall knowledge of ROS, as well as new results based on the hypothesis, is being expressed in these experiments, it is evident that further investigation into the hypothesis is necessary.

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P3-6: AFRICAN AMERICAN AND LATINA BREAST CANCER PATIENTS WITH COMORBID DISEASES SUCH AS HYPERTENSION AND DIABETES HAVE POOR OUTCOME. THIS MAY BE LINKED TO IGF-1 GENE POLYMORPHISMS

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Background: Epidemiological studies have shown that African American (AA) and Latina women with breast cancer have poor outcomes. The poor outcome is significantly associated with comorbid conditions, especially diabetes and hypertension. In our study, we have postulated that African American and Latina women with comor-

bid diseases will have a poor outcome, and this may be influenced by the IGF-1 axis. IGF-1, insulin-like growth factor 1, is a potent mitogen that regulates growth of breast epithelial and stromal cells. Conversely, IGFBP-3, insulin-like growth factor binding protein 3, inhibits IGF-1 action and contributes to cellular apoptosis. We have hypothesized that IGF-1 and IGFBP-3 promoter polymorphisms may have an association with breast cancer and/or comorbidities in African American and Latina women.

Methods: We studied 286 subjects (106 AA and 180 Latina), which included 111 cases and 175 controls. DNA was extracted from blood samples and IGF-1 and IGFBP-3 genotyping was performed by PCR-GenScan and PCR-RFLP methods, respectively.

Results: Our results demonstrated that 47% of breast cancer patients have comorbidities (diabetes and/or hypertension) as compared to 26% of controls ($p \leq 0.0001$). This study also found a significant difference in the distribution of IGF-1 genotypes between the two ethnic groups with African Americans having the greatest occurrence of the non-19/non-19 genotype at 19% versus 3% of the Latina subjects ($p \leq 0.0001$). Also, there was a significant association ($p = 0.02$) between the non-19/non-19 genotype in cases (15%) versus controls (5%). The association of comorbidities with the IGF-1 non-19/non-19 genotype was also significant ($p = 0.042$). No significant association was found with the IGFBP-3 polymorphism and cancer or comorbidities. There was however, a significant difference ($p \leq 0.0001$) in the distribution of the IGFBP-3 genotype with Latina patients having the highest occurrence of the CC genotype (51%) versus African Americans (32%).

Conclusions: Our study, demonstrates a significant association of IGF-1 gene polymorphisms and breast cancer. The data suggest a link with existence of comorbid diseases. It is of particular significance for African American patients since the greatest association was observed between the IGF-1 genotype, hypertension, and breast cancer in this cohort.

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P3-7: BREAST CANCER EPIDEMIOLOGY IN PUERTO RICO

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While breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among females in Puerto Rico (PR), rates of breast cancer are considerably lower than the rates in the continental United States. At the same time, the rate of breast cancer has been climbing far more rapidly among Puerto Ricans than among women in the United States. The reasons for these differences are not known but could be due to changing lifestyles, environmental exposures, or genetic factors that differ from those in the mainland United States. Exploration of breast cancer risk in a population with changing rates of disease is of particular interest because of greater variability in exposures that may provide insight into disease risk for the better-studied populations where most, if not all, women have high exposures.

We are in the process of establishing a case control study of breast cancer in PR and a program to train breast cancer researchers in PR. The case control study will enroll women ages 30–79 who are residents of the San Juan metropolitan area. Cases will be women with incident, primary, pathologically confirmed breast cancer with no history of previous cancer other than non-melanoma skin cancer; controls will be frequency-matched on age and randomly selected from women residents of the same geographical area.

We will examine adult and childhood factors in relation to risk of breast cancer in this understudied population of Puerto Rican women. Our aims are: (1) to examine dietary risk factors. We hypothesize that factors associated with more traditional diet will be less common in cases than in controls. We will examine risk factors in relation to breast cancer and also in relation to tumor characteristics (e.g., estrogen and progesterone receptor status). (2) To examine other, more established risk factors in Puerto Rican population (e.g., lifetime weight gain, other dietary factors, physical activity, alcohol consumption, and reproductive history). We hypothesize that, in spite of the ecological differences in population characteristics and disease trends, the associations of these factors will be similar to what has been observed in other, better-studied populations. We will examine risk factors in relation to breast cancer and also in relation to tumor characteristics. (3) To examine other factors related to early life exposure including birth weight, adult height, childhood diet, physical activity environmental factors, and residential history as a proxy for environmental exposure. We will examine risk factors in relation to breast cancer and also in relation to tumor characteristics.

The overall training goal is to develop a team of independent investigators with the necessary skills to develop a program of breast cancer research in PR and to obtain funds and support for that research. To accomplish this goal, researchers from the UPR will obtain formal training in cancer epidemiology and participate in the design and conduct of the population-based case control study. Formal training will include

coursework, workshops, interactions with experts, and with the primary collaborating mentor.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0329.

P3-8: CHARACTERIZATION OF IGF-1 (CA)_n REPEAT AND IGFBP3 POLYMORPHISMS IN AFRICAN AMERICANS AND HISPANICS WITH BREAST CANCER

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Background: Insulin-like growth factor-1 (IGF-1) is a peptide growth hormone that is intimately involved in the proliferation of normal and malignant breast epithelial cells. The actions of IGF-1 are regulated by a family of binding proteins (IGFBPs). IGFBP3 is the most abundant in circulation and its binding to IGF-1 regulates both breast epithelial and stromal cell proliferation and apoptosis. We had previously demonstrated that high plasma levels of IGF-1 and low serum levels of IGFBP3 are associated with an increased risk of breast cancer and with poor prognosis in AA women. IGF-1 and IGFBP3 both have an important polymorphic site in their promoter region, (CA)_n repeat and A/C, respectively, which may affect their expression pattern. The AA cohort is known to be a high risk group for poor survival from breast cancer. The biological or genetic factors that could contribute to the disparity are poorly understood in minority populations compared to Caucasians. In our current study, we hypothesized that CA repeat polymorphisms in IGF-1 and -202A/C polymorphism in IGFBP3 may influence the risk of breast cancer development and perhaps in the prognosis of the disease.

Methods: In our case-controlled study, we screened a cohort of 455 subjects from minority population for IGF1 and IGFBP3 polymorphisms. IGF1-CA repeat polymorphism was analyzed by PCR-GenScan method while -202A/C IGFBP3 polymorphism was detected by PCR-RFLP (restriction fragment length polymorphism) method.

Results: The (CA)₁₉ repeat allele of IGF1 was the most common allele in both cases and controls. Non-19/non-19 repeat genotypes were more frequent in AAs as compared to the Latina group. In a multivariate analysis of IGF-1 genotypes, AAs showed a 3-fold increased risk (OR=3.2 at 95%CI 0.9-11.6, $p=0.07$) with non-19/non-19 genotype. In comparison, Latinas had more than 9-fold increased risk (OR=9.6 at 95%CI 1.4-52.0, $p=0.02$). The IGFBP3 polymorphism pattern showed a significant difference between the two ethnic groups (in controls and cases). Latinas had a greater distribution of the CC genotype. However, there was no significant association with breast cancer risk.

Conclusion: Our study suggests an important association of the IGF-1 non-19/non-19 CA repeat with breast cancer. Further studies with a larger number of study subjects will help us to validate the role of IGF-1 and IGFBP3 polymorphisms in minority populations.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0340 and National Cancer Institute.

P3-9: COMMUNITY UNINSURANCE AND BREAST CANCER SCREENING

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This HBCU/MI Partnership Training Award established a research and training collaborative partnership between the Institute for Population Health Policy (IPHP) at the University of Texas-Pan American—a Minority Institution—and the Leonard Davis Institute of Health Economics (LDI) at the University of Pennsylvania (Penn). The main objective of the UTPA-Penn breast cancer research/training partnership is to develop a competitive and successful breast cancer research program at UTPA that focuses on cancer control and population sciences.

One of the projects being conducted under this UTPA/Penn partnership is the study of community uninsurance and breast cancer screening. About 47 million people in the United States do not have health insurance coverage, and uninsurance has been linked to lower mammography screening rates and delays in the detection of breast cancer. Uninsurance may also be associated with reduced care for the surrounding insured population if high levels of community uninsurance create financial stress on local health care systems. Mammography services may be particularly sensitive to community insurance rates because mammography screening facilities are financially sensitive to changes in the demand for their services. Mammography screening facilities must operate near full capacity to cover their fixed costs. Mammography use may

also be particularly sensitive to community uninsurance because of the growing shortage of breast radiologists and certified mammography technologists.

The objective of this study is to examine whether low rates of community insurance are associated with reduced use of mammography screening for both insured and uninsured adult women 40 to 69 years of age (N=13,438). Survey data from the 2000–2001 Community Tracking Study Household Survey (CTSHS) is utilized to estimate multilevel logistic regression models of the determinants of mammography screening. Multilevel statistical modeling accounts the contextual effects of local-level uninsurance through the inclusion of community-specific random effects.

The main hypothesis of the study is that both insured and uninsured women residing in communities with a relatively large uninsured population are less likely to undergo mammography screening than if they resided in communities with a relatively small uninsured population. Women ages 40 to 69 were less likely to report that they had a mammogram within the last year if they resided in communities with a relatively high uninsurance rate, even after adjusting for other factors. After adjusting for individual insurance and other factors, a 10 percentage-point decrease in the proportion of the local insured population is associated with a 17% (95% CI=13-21) decrease in the odds that a woman aged 40–69 will undergo mammography screening within a year. The probability of mammography screening falls by 1.3% for every 10% increase in community uninsurance.

This study shows that uninsurance within a community is associated with reduced mammography use among women in this community regardless of whether these women are themselves insured or not. These results are important because they demonstrate that uninsurance is not just a problem for the uninsured, but it is a dilemma for everyone in society regardless of individual health insurance status.

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P3-10: ABSTRACT WITHDRAWN

P3-11: PROTEOMIC CHARACTERIZATION OF MCF7 HUMAN BREAST CANCER CELLS RESISTANT TO TNF- α AND CHEMOTHERAPEUTIC DRUGS

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Despite intense studies on the mechanisms of chemotherapeutic drug resistance in human breast cancers, few reports investigate systematically the possible mechanisms responsible for the resistance to TNF- α , a sensitizing agent in tumor chemotherapy. In this study, 2-DE and LC-MS/MS approaches were used to compare the differences of protein expressions between an MCF-7 breast cancer cell line resistant to TNF- α and its parent MCF-7 cells sensitive to TNF- α . Proteomic analysis identified seven protein spots that differed significantly in abundances in the two cell lines. The differential expressions of these protein spots were verified with both semi-quantitative RT-PCR and real-time RT-PCR assays, and the genes e-cadherin, β -catenin, *snail*, *slug*, and δ -*efl* involved in the epithelial-mesenchymal transition (EMT) were detected simultaneously. The study suggests that upregulations of vimentin (VIM), heat shock 70 kDa protein 4 (HSPA4), glutathione S-transferase P (GSTP1), and creatine kinase B-type (CKB), and downregulations of keratin 8 (KRT8), keratin 19 (KRT19), and glutathione S-transferase Mu 3 (GSTM3), and the epithelial to mesenchymal transition are possibly related to TNF- α resistance in MCF-7 cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0557.

P3-12: DESIGN OF A PEPTIDE TO DISRUPT THE INTERACTION BETWEEN Hsp90 AND p23

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The heat shock response mediated by the heat shock protein Hsp90 and its co-chaperone p23 allows cells to survive mutations and may contribute to carcinogenesis. The interaction between Hsp90 and p23 is a potential target for drug therapy. Geldanamycin and herbimycin are known to inhibit Hsp90 activity. Preliminary results show that mice embryonic fibroblasts that are null for p23 are more susceptible to the toxic effects of geldanamycin and herbimycin. We are currently using molecular modeling to design a peptide that will bind specifically to p23 and prevent p23 from binding to Hsp90. Such a peptide introduced into the cells would make them functionally p23 null. This would make the tumor cells more susceptible to the toxic effects of geldanamycin and herbimycin.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0557.

P3-13: INTERACTIONS OF ESTROGENIC PESTICIDES ON BREAST CANCER CELL GENE EXPRESSION EVALUATED WITH A CANCER FOCUSED PCR ARRAY**Thomas Wiese,¹ Huiming Li,¹ H. Chris Segar,¹ and Steven R. Hill²**¹Xavier University of Louisiana, New Orleans and ²Tulane University

A number of pesticides have been shown to stimulate estrogen receptor (ER) mediated proliferation and induce gene expression in breast cancer cells in culture. Thus, exposure to such compounds could be a contributing factor in the progression of breast cancer. We have previously described the estrogen regulated proliferation and reporter gene activity of DDT isomers and metabolites as well as of methoxychlor and its primary metabolite HPTE. In addition, we have shown that some organophosphate pesticides may potentate this estrogen activity in particular binary mixtures. The goal of this study is to characterize the interactive effects of binary mixtures of pesticides and metabolites with estrogen activity on breast cancer cell gene induction. Most

published characterizations of the hormone activity of pesticides in breast cancer cells have measured only the effects of single, pure compounds. Considering that real life exposure includes multiple isomers and/or the production of metabolites, it is of interest to examine what effect a mixture of these isomers and metabolites may have on genes related to breast cancer etiology and progression. MCF-7 cells were treated with mixtures of estradiol and one of the test pesticides as well as various mixtures of two pesticides, including combinations of one DDT and one of the organophosphate pesticides fenitrothion, methylparathion, or parathion. RNA collected from these cultures was then used in Breast Cancer and Estrogen Receptor Signaling PCR Arrays from SuperArray. Analysis of these arrays in relation to each other and arrays from controls (blank, estradiol, single pesticides) are presented with highlight of novel gene expression patterns resulting from binary pesticide mixtures.

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TRANSLATIONAL RESEARCH AWARDS I

Poster Session P4

P4-1: RAPID INTRA-OPERATIVE DIAGNOSIS OF SENTINEL LYMPH NODE METASTASIS IN BREAST CANCER UTILIZING SCANNING ELASTIC SCATTERING SPECTROSCOPY

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Introduction: Sentinel lymph node (SLN) biopsy is the standard of care for lymphatic staging in early breast cancer. Intra-operative detection of SLN metastases avoids a second operation in SLN positive patients. Current intra-operative diagnosis techniques using touch imprint cytology (TIC), frozen section histology and newer reverse transcriptase polymerase chain reaction (RT – PCR) assays are resource intensive, expensive, time consuming and require experienced personnel to report results. Optical biopsy utilizing Elastic scattering spectroscopy (ESS) is an instant, inexpensive alternative. ESS is an optical technique, which interrogates tissue by short pulses of broadband white light using statistical techniques to discriminate between spectra from normal and abnormal tissue.

Methods: After institutional review board approval, a diagnostic algorithm was developed from a training set of spectra obtained from totally metastatic and completely normal lymph nodes. Spectra were analyzed by principal component analysis and linear discriminant analysis, calculating a canonical score for each spectrum. The canonical scores were plotted as a receiver-operator curve (ROC) and a threshold score of 1 was determined to have the maximum sensitivity and specificity for diagnosis of metastases. In the second stage of the study, a prototype ESS scanner was built for systematically scanning a 10x10mm (0.5mm step size) area of the cut surface of the node resulting in 400 spectra. The spectra obtained were statistically analyzed using the diagnostic algorithm to produce a real time color image of the scanned area of the SLN. An image with ≥ 4 contiguous positive pixels was considered positive for metastases. The results were correlated with the final histopathology.

Results: 117 nodes were scanned from 71 patients. 22 early scans were excluded for technical reasons resulting in 95 satisfactory scans. For detection of cancer, the sensitivity was 74% and specificity 96%.

Conclusions: The initial results using the ESS scanner are comparable to current intra-operative diagnostic techniques. The significant advantages to conventional pathological techniques include minimal running costs, no tissue preparation or destruction and no requirement for an expert pathologist for interpretation. It has potential universal availability for real time diagnoses in the operation room. The development of this prototype scanner enables systematic scanning for detection of small metastatic deposits. Refinement of hardware and software with faster scanning of multiple sections of nodes could reduce the false negative results, improving sensitivity further.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8343 and Hamamatsu Photonics.

P4-2: INITIAL CALLBACK RATES FOR CONVENTIONAL AND DIGITAL BREAST TOMOSYNTHESIS MAMMOGRAPHY COMPARISON IN THE SCREENING SETTING

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Purpose: Measure the callback rate for digital breast tomosynthesis (DBT) compared to callback rates for conventional 2D, 4-view digital mammography (CM) in a screening population Look at callback rates by ACR density in this group.

Method and Materials: A total of 2,035 women (ages 35–93, mean 58.5) accrued to date into the NCI-NIB 3,000-women DBT screening trial begun in August 2005 using the GE Gen 1.5 DBT clinical prototype. One MLO view per breast was acquired as 15 low-dose projections over a 40-degree arc in 14–23 seconds using standard clinical compression. Eight-iteration maximum-likelihood expectation-maximization (MLEM) reconstruction yielded one slice per mm of breast thickness rendered for review on an ultra-performing review workstation integrated into the PACS. CMs were read in the usual clinical workflow with results recorded on case forms by 10 clinical staff radiologists (4–33 years of experience). DBT studies were read both with (P) and without previous study reference (noP) by two expert breast radiologists (10–33 years of experience). Each of the four readings was in turn recorded. The DBT reading without priors were timed.

Results: ACR BIRADS radiographic density, age, and compressed thickness (22 to 70 mm, mean 54 mm) were cross-tabulated with ACR outcome score (0, 1, 2, 3, 4, 5) for each of the reading conditions. Overall, callback rates were distributed DBT-P-114 (5.1%), DBT-noP-258 (11.6%), CM-noP-287 (12.9%), CM-P-181 (8.1%) out of 2,083 cases. The DBT-P density-callback rates were 1–3.8%, 2–4.9%, 3–5.0%, 4–6.3%. For comparison, the CM-P density-callback rates were 1–5.6%, 2–6.1%, 3–9.3%, 4–8.3%.

Conclusions: DBT markedly reduced the callback rate in a 2,035-women sample of the screening population from 8.1 to 5.1%, a 37% reduction. Callback rates appear similar across ACR radiographic density 1–4.

Clinical Relevance: DBT improves tissue visualization by removing overlying and underlying tissue to permit improved lesion characterization that can reduce callback rate in a screening setting.

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P4-3: ENDOCRINE THERAPY OF BREAST CANCER

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Background: Currently, we fail to predict endocrine responsiveness in ~66% of ER+/PgR-, 55% of ER-/PgR+, and 25% of ER+/PgR+ tumors. Overall, only 50% of ER+ tumors respond to endocrine therapy. A more accurate predictor of endocrine responsiveness would have widespread clinical use, allowing women and physicians to make more individualized and appropriate treatment decisions.

Methods: Our primary goal is to build better predictors of responsiveness to anti-estrogens versus aromatase inhibitors. A secondary goal, using different analysis methods of the same data sets, is to extract meaningful mechanistic insights into the molecular pathways driving endocrine responsiveness in breast cancer. Thus, we are in the process of completing two molecular profiling clinical studies of tamoxifen versus aromatase inhibitors (one retrospective and one prospective study). We have definitive clinical outcomes data for the retrospective study with follow-up in excess of 10 years for almost all cases and date and site of recurrence data (e.g., local, regional, and/or distal recurrence). We have clinical and pathological response data for the prospective study, and we continue to collect survival data. We routinely collect RNA for microarrays, DNA for SNPs, and protein for Western analysis—this award is primarily to perform the microarray analyses. All specimens are subjected to histopathological review. Total RNA is extracted from tumor-enriched samples using Trizol and cleaned with the RNeasy MinElute Cleanup Kit. RNA quality is checked on RNA Nano Chips with the Agilent 2100 bioanalyzer from Agilent Technologies. We use Affymetrix U133 plus 2 GeneChips and validated standard operating procedures for tissue acquisition, processing and extraction, and for specimen labeling and hybridization. We also continue to develop novel methods for data normalization and analysis.

Results: To date, we have obtained almost all of the required specimens from both the retrospective and prospective studies. Thus, we have over 481 breast cancer specimens, including those from the prospective neoadjuvant study, for a total of n=414 cases. As might be expected, these specimens mostly represent patients treated with TAM (n=378 cases). Our studies are ongoing, and we have not yet completed data analysis. However, we have generated and independently validated several novel computational methods including those for data visualization (e.g., VISDA), for normalization (e.g., iterative nonlinear regression), for building predictive models (e.g., optimized multilayer perceptrons), and for gene network modeling (e.g., multilevel ICA and motif analysis methods). A pilot analysis of the specimens arrayed to date is currently in progress.

Conclusions: Evidence has begun to accumulate suggesting that an aromatase inhibitor may be a more effective first-line endocrine therapy for some breast cancer patients than tamoxifen. These data have generated considerable interest and controversy, in part because unlike TAM, there are very few data from long-term studies with aromatase inhibitors where definitive survival data are available. Our study could provide new methods for the more effective targeting of specific endocrine therapies to individual patients. We also anticipate gaining innovative insights into how breast cancers become resistant to endocrine therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0570.

P4-4: THE MONOCLONAL ANTIBODY 7.16.4 SEQUENCED WITH CYCLOPHOSPHAMIDE-MODULATED VACCINATION MAXIMIZES TUMOR IMMUNITY AND TUMOR-FREE SURVIVAL IN TUMOR-BEARING TOLERANT *neu-N* MICE

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Johns Hopkins University School of Medicine

HER-2/*neu* (HER-2) is overexpressed in up to 25% of human breast cancers and portends a poor prognosis. HER-2 is also a target of the antitumor immune response in breast cancer patients. Notably, the humanized monoclonal antibody Trastuzumab can result in the clinical remission of treatment-refractory metastatic breast tumors that overexpress HER-2 and improves clinical outcomes for early breast cancer as well. *neu-N* mice transgenic for the rat protooncogene *neu* spontaneously develop mammary tumors and are profoundly immune-tolerant to HER-2. We previously showed that a low dose of cyclophosphamide (CY) given 1 day prior to vaccination can eliminate the negative influence of regulatory T cells in *neu-N* mice, enabling the cure of up to 25% of tumor-bearing mice. We also showed that HER-2-specific monoclonal antibodies combined with vaccine could augment HER-2-specific CD8⁺ T cell immunity and tumor-free survival in *neu-N* mice. We hypothesized that both abrogating the influence of regulatory T cells with CY and augmenting antigen processing and presentation with the HER-2-specific MAb would maximize the tumor rejection response in tumor-bearing animals. As a model for clinical translation, we used the Trastuzumab-like murine MAb 7.16.4. The administration a low dose of CY with weekly 7.16.4 MAb to *neu-N* mice with pre-established tumors resulted in tumor-free survival rates of about 10% at 50 days after tumor challenge. Treating tumor-bearing *neu-N* mice with HER-2⁺, GM-CSF-secreting vaccination added to CY + 7.16.4 MAb therapy increased the tumor-free survival rate to 55%. The effect was associated with an increase in HER-2-specific CD8⁺ T cells, anti-tumor antibodies, and tumor cell apoptosis. Preliminary data suggests that modulating vaccination with 7.16.4 might induce a more heterogeneous HER-2-specific T cell response. Based on these data, we have designed a clinical trial testing of CY-modulated vaccination in the setting of weekly Trastuzumab therapy in patients with advanced HER-2⁺ breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0485; American Cancer Society; Genentech, Incorporated; and Cancer Treatment Research Foundation.

P4-5: APPLICATION OF PTC299 IN BREAST CANCER: PRECLINICAL AND CLINICAL STUDY RESULTS

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Background and Objectives: VEGF is a clinically validated mediator of tumor angiogenesis. PTC299 has been identified as an orally bioavailable, small molecule that selectively inhibits tumor overproduction of VEGF through processes dependent on the 5' untranslated region of the VEGF mRNA. In multiple human tumor xenograft models, PTC299 selectively reduces elevated human VEGF levels in tumor and in circulation, decreases tumor microvessel density and perfusion, and substantially slows tumor growth. PTC299 may provide breast cancer patients with a novel therapeutic option.

Methods: The effects of PTC299 on VEGF levels and tumor growth were assessed in mouse xenograft models of human breast cancer, including estrogen-responsive models (MCF-7 and T47-D) and an estrogen-insensitive model (MDA-MB-468-1). The data from these studies, together with data from in vitro efficacy and animal toxicology studies, subsequently supported the initiation of Phase 1 single-dose (SD) and multiple-dose (MD) studies in healthy human volunteers. The SD study included 5 cohorts of 8 subjects (4 male:4 female) per cohort in a dose-ranging stage and 12 subjects (6 male:6 female) in a fed-fasting crossover stage. In the MD study, 4 cohorts of 8 subjects (4 male:4 female; 6 PTC299:2 placebo) per cohort were randomized in a double-blinded manner to receive BID or TID dosing for 7 days. Clinical observations, laboratory testing, and blood sampling for PK and circulating VEGF were performed.

Results: PTC299 prevented the growth of MCF-7 xenografts and reduced the growth rate of T47D xenografts by 64% compared to vehicle. PTC299 treatment of mice bearing MDA-MB-468-1 xenografts decreased human tumor and plasma VEGF levels by 61% and 75%, respectively; impeded growth such that tumors were 65% smaller than the vehicle-treated tumors after 8.4 days; and increased the time for the mean tumor volume to reach 1000 mm³ from 8.4 days to 27 days (p<0.05) relative to vehicle administration. In the Phase 1 SD study, 52 subjects (18 to 55 years old) were

enrolled (40 at 5 dose levels and 12 to assess food effects). In the MD study, 32 subjects (31 to 78 years old) were enrolled (24 at BID dosing and 8 at TID dosing). Adverse events included headache, dizziness, nausea, vomiting, and stomach discomfort (all Grade 1) and diarrhea (Grade 2). No bleeding, clotting, hypertension, or proteinuria occurred. C_{max} and AUC increased proportionally to dose with a 2-fold increase over 7 days, exceeding target through plasma concentrations active in xenograft models. There was an ~40% increase in C_{max} but no change in AUC with food. As expected from preclinical data, physiologic circulating VEGF levels were not perturbed.

Conclusions: PTC299 reduces tumor-derived VEGF production and tumor growth in breast cancer xenografts. Based on preclinical and Phase 1 clinical data, an Investigational New Drug application has been filed with the U.S. Food and Drug Administration, and a Phase 1b/2 study in women with advanced breast cancer has been initiated.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0629.

P4-6: ANTIMETASTATIC THERAPY WITH WX-UK1 AND WX-671 FOR THE TREATMENT OF BREAST CANCER: PHASE I RESULTS AND PHASE II DESIGN

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Distant metastases rather than the primary tumor itself remain the principal cause of death in patients with malignant solid tumors. uPA (urokinase-type plasminogen activator) and its inhibitor PAI-1 play a key role in tumor invasion and metastasis. At this time uPA and PAI-1 are the only biomarkers that have been validated by the European Organization for the Treatment and Research of Cancer at the highest level of evidence (LOE I) with regard to their clinical utility in breast cancer prognosis. In the recently published "ASCO 2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer," uPA and PAI-1 were added to the list of recommended tumor markers, the system being considered a promising target for therapeutic studies.

WX-UK1 is an active site competitive inhibitor of serine proteases with an inhibition constant (K_i) for human uPA in the sub-micromolar range. In preclinical animal tumor models, parenterally administered WX-UK1 and its orally administered pro-drug WX-671 reduce the growth rate of implanted tumors, inhibit their invasion into nearby lymph nodes and the spread to distant target organs (metastasis).

WX-UK1 in combination with capecitabine has been investigated in a phase I study to determine the safety, tolerance, maximum tolerable dose (MTD) and pharmacokinetics (PK) in patients with advanced malignancies. WX-UK1 was infused once a week for 3 consecutive weeks and capecitabine was given orally twice a day for 14 days. The study enrolled 38 patients receiving 7 different dose levels of WX-UK1 between 0.3 and 3.5 mg per kg body weight; capecitabine was dosed at 1000 mg/m² bid.

No dose limiting toxicities were observed during the entire treatment period, and no MTD could be identified in this study. This demonstrates that the combination was well tolerated by all patients for the duration of the study. The MTD is higher than the highest dose used in this study, 2.8 mg/kg. WX-UK1 has dose linear PK properties over the entire dose range and there is no significant drug-drug interaction between capecitabine and WX-UK1.

No definitive conclusions regarding efficacy could be drawn as the study population was small. However some patients showed a partial response during the study. This is encouraging as these patients all had advanced, metastatic tumours, and no standard efficacious treatment existed for them.

In a subsequent phase II study WX-671, the oral pro-drug of WX-UK1 will be used instead of WX-UK1, as it has been demonstrated that (A) daily oral WX-671 administration delivers levels of the active metabolite WX-UK1 equivalent to those achieved with intravenous WX-UK1 itself; (B) repeated WX-671 dosing is safe and well tolerated in clinical trials; and (C) WX-671 in capsule formulation is stable.

This phase II trial will be a two-arm, double-blind, multi-center, randomized study of the combination of oral WX-671 plus capecitabine vs. capecitabine monotherapy in first-line HER2-negative metastatic breast cancer. Each arm will enroll 50 patients. The primary objective is to evaluate the efficacy of the combination of WX-671 and capecitabine compared to capecitabine monotherapy, as assessed by comparison of progression-free survival rates.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0634.

P4-7: PHASE II STUDY OF A HER-2 NEU PEPTIDE-BASED VACCINE PLUS CONCURRENT TRASTUZUMAB FOR PREVENTION OF BREAST CANCER RELAPSE

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Background: Breast cancer relapse after optimal therapy is common in patients with HER-2/neu (HER2)-positive tumors and is likely due to residual microscopic disease. One approach to the eradication of residual subclinical disease is tumor vaccines that generate tumor-specific T cell immunity, specifically, memory T cells capable of eradicating tumor antigen-bearing cells over an extended period of time. Immunity against the intracellular domain (ICD) of the HER2 protein correlates with antitumor responses in animal models. Patients with HER2+ cancers can be immunized to the HER2 ICD using peptide-based vaccines. Moreover, trastuzumab, a standard therapy for HER2+ patients, increases the activity of HER2-specific T cells in vitro. Thus, concurrent administration of trastuzumab with HER2 vaccines may enhance the generation of HER2-specific CD4+ and CD8+ T cell responses and potentially translate into improved overall survival (OS) for advanced-stage patients. We have initiated a Phase II study to examine the OS, safety, and immunogenicity of an HER2 ICD peptide-based vaccine when administered concurrently with trastuzumab to patients with Stage IIIB/IV breast cancer.

Methods: A total of 52 subjects with HER2+ Stage IIIB or IV breast cancer who are currently on maintenance trastuzumab and have been treated to a state of no evidence of disease (NED) or stable bone-only disease (SBD) with trastuzumab alone or in combination with chemotherapy will be enrolled. Subjects are enrolled within 6 months of initiating maintenance trastuzumab and must have a normal baseline MUGA scan. The HER2 ICD peptide vaccine is composed of 3 HER2 Class II epitopes (p776-790, p927-941, and p1166-1180), and given intradermally with GM-CSF as adjuvant every 30 days for a total of 6 vaccines. The primary end point is evaluation of OS at 2 years compared to historical controls. Secondary end points include immunogenicity and safety. HER2-specific immune responses are assessed by IFN- γ ELISPOT at baseline and after vaccine 3 and 6. Toxicity is assessed at baseline prior to each vaccine and at follow-up.

Results: A total of 9 subjects have been enrolled to date, 7 Stage IV (5 NED and 2 SBD) and 2 stage IIIB. Median time from last chemotherapy was 4 months (range 1–11). A total of 7 subjects have completed 6 vaccines, and a total of 51 vaccinations have been given. Toxicities observed are grade I and II (82% and 17%, respectively) with the most common being fatigue (9%), myalgia (8%), leukopenia (8%), and lymphopenia (8%). There have been no related Grade 3 or 4 toxicities and specifically, no cardiac toxicities. At interim analysis, 3 of 4 patients have developed T cell immunity, defined as HER2-specific T cell precursors:PBMC) to p776, p927, and p1166. The median precursor frequency to p776 was 1:3,937 (range 1:1,051–1:20,000), to p927 was 1:1,574 (range 1:727–1:32,467), and to p1166 was 1:1483 (range 1:610–1:37,453). Interim survival data and complete immunologic analysis will be presented on patients enrolled to date.

Conclusions: Early data suggest that subjects with HER2+ stage IIIB and stage IV cancer can be safely immunized with an HER2 peptide vaccine while receiving concurrent trastuzumab. Additionally, the approach is immunogenic, generating significant levels of HER2-specific T cell immunity. Accrual continues and long-term follow-up is ongoing for survival benefit analysis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0561.

P4-8: A POLYVALENT, SHED ANTIGENS, VACCINE FOR BREAST CANCER

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We have developed a novel strategy to construct cancer vaccines that addresses a fundamental problem that limits the effectiveness of current vaccines: relevant tumor antigens that mediate protective immunity are unknown. It is based on constructing the vaccine from tumor antigens shed into culture medium by allogenic cancer cells. This results in a broadly polyvalent vaccine that contains numerous tumor antigens. This both enhances the chances the vaccine will contain relevant antigens and circumvents the antigenic heterogeneity of cancer cells. This strategy has been validated in melanoma, where a shed antigen vaccine for this cancer has proven safe to use, able to stimulate both antibody and CD8+ T cell responses to a broad spectrum of melanoma antigens, and doubled recurrence-free survival in a double-blind and placebo-controlled trial.

We have now shown this approach can be applied to make a similar vaccine for breast cancer. We have been able to establish multiple lines of human breast cancer cells in long-term, serum-free culture. We have shown these cells express multiple breast cancer-associated antigens including, but not limited to, MAGE-1, MAGE-3, CEA,

HER2/neu, and NK-1-C3. We have selected a panel of these cells to be used for vaccine construction, based on the cells expressing different patterns of these antigens, to increase the spectrum of antigens present in the vaccine. We have shown that these antigens are released into culture medium, indicating the vaccine can be made from shed antigens. A pilot lot of this vaccine has been made.

This work indicates that a polyvalent shed antigens vaccine for breast cancer can be made. The advantages of such as vaccine are that: (1) it contains a broad spectrum of tumor antigens, (2) the antigens are partially purified as they are separated from the bulk of cellular material which is not shed, and (3) the antigens are more likely to be biologically relevant as they are expressed on the surface of tumor cells where they can be seen and interact with host immune defense mechanisms. The effectiveness of this approach is validated by the results of extensive clinical trials in melanoma.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9320 and New York University.

P4-9: RAPID TRANSLATION OF A NOVEL AND POTENT VACCINE COMBINATION IN HER2+ HERCEPTIN® REFRACTORY METASTATIC BREAST CANCER

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This clinical translational research award seeks to evaluate a novel viral vector vaccine combination for testing in trastuzumab-refractory breast cancer patients. Both viral vectors express the human HER2 gene and individually promote potent immune responses mediated by cellular and antibody-mediated immunity. One is an adenovirus construct (Ad-HER2), and the other is an alphavirus VPR construct (VPR-HER2). When used in combination, the effectiveness of the vaccine is significantly enhanced in animal models. In particular, we have demonstrated that the vaccine elicits potent T cell and antibody responses and that these antibody responses are capable of both inhibiting proliferation and directly killing trastuzumab-refractory human breast cancer cells.

Our Specific Aims:

1. To perform preclinical studies, conduct GMP manufacture of Ad-HER2 and VPR-HER2 vectors, and complete the IND package for FDA submission.
2. To perform two Phase 1 clinical trials, one for each vector, with the primary end point being safety.
3. To conduct a Phase 1/2 clinical trial combining the two vectors in a heterologous vector prime-boost regimen. Primary end point for the Phase 2 study is clinical benefit.

Progress to Date: In the first year of the grant, we held a pre-IND meeting with the FDA and received important feedback on the agency's stance on the use of the HER2 gene as a vaccine. In particular, the FDA requested that we perform further preclinical studies on the HER2 transgene sequence to demonstrate lack of "oncogenic potential" prior to selection of the final HER2 transgene sequence for our clinical-grade vectors. We have completed these extensive studies and are in the final stages of downselecting the optimal transgene sequence for each vector, which includes testing pilot yields of virus for production scale up. VPR-HER2 will be manufactured under contract by AlphaVax Human Vaccines Inc., our collaborator on this proposal. The Ad-HER2 vaccine manufacture will be contracted to SAFIC Pharma (formerly Molecular Medicine BioServices), who have licensed the Crucel PerC6 packaging line that has a superior safety profile. Manufacturing of the vectors is scheduled to begin in Q2/2008.

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P4-10: A MIMIC OF TUMOR REJECTION ANTIGEN-ASSOCIATED CARBOHYDRATES MEDIATES AN ANTITUMOR CELLULAR RESPONSE

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Background and Objectives: Carbohydrates are the most abundantly expressed auto-antigens on tumor cells, and consequently, they are perceived as viable targets for immunotherapy. While pure carbohydrate antigens elicit diminished immune responses because of their T cell independent nature, conjugate vaccine technology has overcome some of the limitations of carbohydrates as vaccine antigens because of the T-dependent help conferred by the protein. Carbohydrate conjugates are characteristic of traditional vaccines inducing Th2-biased immune responses with a high titer of antibodies and almost without a significant induction of cell-mediated immunity against the respective antigen. Consequently, new formulations or alternative ways to augment carbohydrate reactive immune responses are being evaluated. We are demonstrating that peptides mimicking breast-associated neolactoseries antigens are capable of activating carbohydrate-specific cellular immune responses and that limit tumor growth in vivo.

Methodology, Results, and Conclusions: To study the outcome of peptide immunization on growth of solid tumors in vivo, we evaluated the antitumor cellular response on established Meth A tumors. BALB/c females were inoculated subcutaneously with Meth A cells and 7 days later treatment was started with the peptide-injected intraperitoneally (3 times at 4–5-day intervals). Immunization moderately affected Meth A sarcoma growth as 6 mice out of 11 immunized were cured. Treatment of animals with IL-12 following peptide immunization, intending to enhance the immune response, was successful as it mediated the complete eradication of established tumors. Treatment of tumor-bearing mice with only IL-12 did not affect tumor growth. We further determined that peptide/IL-12 combination therapy is highly effective even in lower doses of IL-12, as 100 µg of daily IL-12 treatment in combining therapy, but not alone, eradicated tumors in 5 mice of 5 challenged. The time of the beginning of immunization and the size of tumor at this time apparently affects the efficacy of immunization. With immunizations started at day 14 or later or treating tumors with mean diameter of larger than 7 mm, the efficacy of immunization dropped significantly. To further confirm a role played by T cells activated by the peptide mimotope, nude mice were transplanted with Meth A cells and injected ip with fresh splenocytes, isolated from cured mice, 10 days later. Immune cells transferred had a dramatic effect on tumor size as by day 15 after transfer tumor was eradicated completely in all 4 mice tested. In a follow-up study, splenocytes were depleted from B cells and enriched for CD4+ and CD8+ cells in vitro and then transferred to tumor-bearing nude mice. Our data indicated a role for CD8+ cells in eradication of tumors. Thus, carbohydrate-mimicking peptides represent a new and very promising tool to increase the efficiency of the immune response to carbohydrates.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0542.

P4-11: CLINICAL TRANSLATION OF A MAMMAGLOBIN-A cDNA VACCINE

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Washington University

Background: Mammaglobin-A has several unique properties that make it an exceptional target for vaccine therapy: (1) Mammaglobin-A is expressed almost exclusively in normal breast epithelium and breast cancer. This exquisite specificity decreases the potential for autoimmunity following vaccine therapy. (2) Mammaglobin-A is dramatically overexpressed in over 80% of primary breast cancers, suggesting that almost all breast cancer patients are likely to be candidates for vaccine therapy. Near universal expression of mammaglobin-A is particularly relevant for the development of vaccine strategies for breast cancer prevention as a successful strategy targeting mammaglobin-A is likely to prevent the majority of breast cancers. (3) Mammaglobin-A appears to be dramatically overexpressed in all stages of disease (noninvasive breast cancer, invasive breast cancer, and metastatic disease). The consistency of mammaglobin-A expression confirms that it is an attractive target for vaccine therapy. (4) Mammaglobin-A is capable of eliciting an immune response in breast cancer patients. In preliminary studies we have demonstrated that a mammaglobin-A cDNA vaccine is capable of eliciting breast cancer immunity in a preclinical model. The overall hypothesis of this project is that a mammaglobin-A cDNA vaccine is safe, feasible, and capable of eliciting an immune response in breast cancer patients. We plan to test this hypothesis in a Phase 1 clinical trial.

Methods: The mammaglobin-A cDNA vaccine for the Phase 1 clinical trial was manufactured under current Good Manufacturing Practice (cGMP) conditions in a production facility at Washington University School of Medicine. Plasmid identity, homogeneity, and purity were determined using a combination of analytical techniques. Novel molecular adjuvants and delivery vehicles have been evaluated in preclinical models using ELISPOT analysis and other measures of immune response.

Results: A protocol for the Phase 1 clinical trial has been approved by institutional and national regulatory agencies. We have successfully manufactured over 1,000 mg of plasmid DNA according to regulatory guidelines. The plasmid DNA is sterile with > 95% supercoiled DNA with extremely low residual endotoxin, RNA, genomic DNA, protein, and organic solvent. Plasmid identity has been confirmed with DNA sequencing and restriction enzyme digestion. These results suggest that the plasmid is suitable for clinical use. Current studies are ongoing to test the ability of novel molecular adjuvants to enhance the efficacy of this cDNA vaccine including the use of multimeric soluble CD40L and encapsulation into novel polymer microparticles.

Conclusions: Mammaglobin-A is a unique breast cancer antigen that is an exceptional target for vaccine therapy. We have successfully manufactured a novel therapeutic under cGMP conditions and are in the process of performing the product release tests required to support an IND application and initiate a Phase 1 clinical trial.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0677.

P4-12: DENDRITIC CELL (DC) BREAST CARCINOMA FUSION CELLS IN CONJUNCTION WITH IL-12, CPG ODN, OR ANTI-CD3/CD28 RESULTS IN THE SELECTIVE EXPANSION OF ACTIVATED TUMOR REACTIVE T CELLS

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We have previously shown that vaccination of patients with DC/breast carcinoma fusions stimulates anti-tumor immune responses in a majority of patients with metastatic disease; however, only a subset demonstrates evidence of tumor regression. To define the factors that limit vaccine efficacy, we examined the biological characteristics of DC/breast carcinoma fusions as antigen presenting cells and the nature of the vaccine-mediated T cell response. Fusion of DCs with breast carcinoma cells results in increased expression of costimulatory and maturation markers, IL-12 and the chemokine receptor, CCR7. Of note, fusion cells also coexpressed IL-10, supporting their ability to deliver stimulatory and inhibitory signals to reactive T cell populations. DC/breast cancer fusions stimulate a mixed T cell response characterized by the expansion of both activated and regulatory T cell populations. Coculture of fusion cells and autologous T cells resulted in a statistically significant increase in CD4+CD25+ T cells that expressed CD69 and IFNγ as well as FOXP3 and CTLA-4, consistent with an activated and regulatory T cell phenotype. To further define the T cell response to DC/breast carcinoma fusions, regulatory and activated T cells were separated by flow cytometric sorting of CD4+CD25^{high} and CD4+CD25^{low} populations. CD4+CD25^{high} but not CD4+CD25^{low} cells uniformly expressed FOXP3. Consistent with a regulatory T cell phenotype, CD4+CD25^{high} cells inhibited the proliferative responses of CD4+CD25+ T cells to tetanus toxoid, anti-CD3, and PHA. These observations suggested that the increased presence of regulatory cells may potentially inhibit the in vivo efficacy of the fusion cell vaccine. As such, we examined several strategies to bias the fusion-mediated T cell response toward activated cells. We found that addition of IL-12, TLR7/8 agonists, CPG ODN, or IL-18 increased the relative presence of fusion-activated T cells and reduced the expansion of fusion-mediated regulatory T cells. We also found that the sequential stimulation with DC/breast carcinoma fusions and anti-CD3/CD28 results in the relative expansion of tumor-specific T cells with an activated phenotype. These strategies thus provide the basis to enhance vaccine efficacy and represent a platform for the successful use of adoptive immunotherapy for patients with breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0487.

CLINICAL MANAGEMENT OF BREAST CANCER

Poster Session P5

P5-1: ABSTRACT WITHDRAWN

P5-2: ROBOTIC MAGNETIC RESONANCE IMAGING GUIDANCE OF LASER TISSUE EXCISION

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Objectives: Magnetic resonance imaging (MRI) provides excellent sensitivity and spatial definition of potentially cancerous tissues within the breast. A robotic system to place biopsy and/or treatment probes has been constructed using largely non-metallic components to locate, sample, and excise cancerous breast tissue. One such probe, a fiber optic laser device, is proposed to allow real-time MRI-guided tissue excision without image degradation caused by typical metallic surgical instruments. Two studies are presented: a characterization of image distortions created by device components placed close to the patient and an evaluation of fiber optic guided lasers as a tissue cutting method for MRI-guided interventions.



Thermal cutting of porcine liver tissue with an 808-nm diode laser

Methods: MR images were taken in a 1.5 Tesla MRI scanner with a phantom placed at the center of a rotating biopsy device stage driven by piezoelectric motors. Image quality was observed with the device in various on-off states. Laser cutting was performed on an ex vivo porcine liver using an 808 nm continuous wave (CW) diode laser. The laser beam was transmitted using bare-tip 600 μ m silica fiber and mounted on a motion control table. Three power settings between 15 and 25 watts (W), and three velocity settings of 0.03, 0.05, and 0.07 inches per second (ips) were used to cut tissue. Cut depth and width and coagulation depth and width were measured for both single-pass and double-pass cuts.

Results: Minimal image distortions were observed when the actuated rotary stage was at rest and in motion. Removing electrical power from the actuator controller caused a reduction in image interference. With one laser pass at 24.80 ± 0.04 W and 0.03 ips, a cut 1.99 ± 0.46 mm deep and 1.39 ± 0.11 mm coagulation were achieved. Response after the first cut at 15.68 ± 0.024 W and 0.07 ips, resulted in 0.89 ± 0.32 mm cut depth and 0.91 ± 0.30 mm coagulation. After the second pass, cut depth was 1.95 ± 0.52 mm with a coagulation depth of 0.95 ± 0.32 mm.

Conclusions: Metallic components, especially power conversion components, contribute interference to the MR images; therefore, shielding and filtering are needed. Metal components may be acceptable for use in an MRI-guided surgical device if interference does not cause gross image distortion. Study indicates the feasibility of cutting tissue using lasers. Changing the power or the exposure time offers the flexibility of changing the depth of cut. Depth of cut can be increased by repeating passes over the tissue. These experiments indicate potential for an MRI-guided tissue excision device.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0397.

P5-3: A PILOT STUDY TO EXPLORE CHANGES IN OVARIAN STROMAL FUNCTION AND ASSOCIATED SYMPTOMS IN PREMENOPAUSAL WOMEN UNDERGOING CHEMOTHERAPY FOR BREAST CANCER

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The quality of life of women diagnosed under age 50 can be adversely affected by premature menopause as a result of chemotherapy agents used to treat breast cancer. Menopause-related symptoms are salient management issues for these women and include hot flashes, vaginal dryness, decreased sexual desire, and negative mood swings. Although estrogen levels and follicular function post chemotherapy have been studied, little is known about how or if androgen levels are affected by chemotherapy and whether androgen changes impact symptoms in breast cancer survivors. The objective of this pilot study is to look for evidence of whether androgen levels are adversely affected in premenopausal women as a result of adjuvant chemotherapy for breast cancer and whether low androgen levels are linked to any undesired symptoms.

Methods: A longitudinal, descriptive design was used. Data, including both subjective and objective measures of menopause-related phenomena, were collected for 20 women who completed blood draws and questionnaires at 4 time periods: baseline (before treatment), mid-treatment, immediate post-treatment, and 6 months later. Self-report questionnaires included the Female Sexual Function Index, Greene Climacteric Scale, the Profile of Mood States (POMS), the cognitive and physical subscales of the Schwartz Cancer Fatigue Scale (SCFS), and a menses diary. Data

analysis included descriptive statistics, specifically, plots of the hormone levels, and change scores over time.

Results: Preliminary data are presented from baseline to immediate post treatment. All of the women age 40 and older did stop menstruating and had an FSH level over 40 IU/L by the end of chemotherapy. Of these women, both estradiol and estrone levels were in the postmenopausal range and androgen concentrations had decreased by at least 35% from baseline, with most levels decreasing 50%. Androstenedione was more consistently lower than bioavailable testosterone concentrations. The women age 28 to 39 retained ovarian function as evidenced by estrogen levels in the premenopausal category, low FSH levels, and some episodic menstrual activity. Several symptoms became progressively worse through treatment and showed a decline of more than 10 points on a 100 point scale by the end of treatment. These symptoms included all domains of sexual functioning (desire, arousal, lubrication, orgasm, satisfaction, and pain), vasomotor symptoms, and vigor and fatigue.

Conclusion: These exploratory data provide evidence that androgens are impacted by chemotherapy treatment. This hypothesis should be confirmed in a larger study. Symptoms, such as vasomotor problems, sexual function changes, and fatigue need to be evaluated in patients throughout treatment and interventions begun early in an effort to prevent negative experiences.

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P5-4: ASSESSING THE NEEDS OF WOMEN WITH ADVANCED (METASTATIC) BREAST CANCER

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Living Beyond Breast Cancer

Introduction/Objectives: As newer treatments become available, women with advanced (metastatic) breast cancer (ABC) are living longer with ongoing needs for information, support, and practical services. An in-depth needs assessment study was done to contribute toward filling in the gaps in information and resources and to guide in the planning of programs and services for this growing underserved population.

Description: Living Beyond Breast Cancer (LBBC) staff, consultants, and an advisory committee developed a 64-question survey. The survey was pilot-tested with the advisory committee, which included oncologists, nurses, social workers, and women with ABC. Their reviews, suggestions, and feedback were integrated into the final survey, which was administered online and on paper to 697 women with ABC, focusing on valued and desired services online, in-person, via print media, and by telephone.

Summary of the Results to Date: Because respondents who completed the paper survey differed demographically and were small in number (11%), they were omitted from this analysis.

Reflecting typical Internet users, the 618 online respondents were relatively younger (70% ages 40–60) and better educated. Most reported living with distant metastases for less than 5 years. Almost all were currently in treatment. Reported current symptoms/side effects were fatigue or weakness (67%), cognitive problems (60%), sexual dysfunction (60%), disturbed sleep (56%), hot flashes (50%), pain (49%), depression (34%), and anxiety (28%). Nevertheless, 44% found maintaining daily routines and activities very easy or easy and only 19% difficult or very difficult.

The desire for information and support was strong among these women. Sixty-seven percent of respondents sought information on ABC at least weekly, 27% daily. Similarly, 62% sought emotional/practical support at least weekly and 30% daily. Top-ranked informational needs focused on treatment options, including clinical trials and symptoms and side effects management. Top-ranked support needs included online and in-person support groups. Top-ranked practical needs centered on referrals for medical care and information/assistance with insurance and disability. Despite frequent information and support seeking, many of these women were unaware of existing services, either online or in their home communities, suggesting a need for tailored information and referral sources and better outreach, publicity, and service coordination. Due to this suggested need, LBBC has developed an annual large-scale conference for women with ABC, teleconferences on topics of special interest to women with ABC, and published a white paper to disseminate the findings of the survey to researchers, consumers, advocacy organizations, and health care professionals working with women with ABC.

Conclusions: This survey offers detailed insights into the experiences, preferences, and unmet needs of women with ABC that will be used both to inform program development at LBBC and to educate other health professionals in the breast cancer community. LBBC will hold another conference for women with ABC in May 2008 and continue to promote and develop targeted programming and publications. Further research on the needs of non-Internet users, especially older women and minorities, is currently being undertaken. Results of the paper survey and a comparison to the Internet survey will be available later in 2008 and will be published.

This work was supported by the Claneil Foundation.

P5-5: TREATING THE WHOLE PATIENT: WHAT BREAST CANCER PATIENTS WOULD LIKE THEIR MEDICAL CAREGIVERS TO KNOW: AN EDUCATIONAL INTERVENTION FOR MEDICAL AND NURSING STUDENTS

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To Life!

Background: To Life!, Inc. is a community-based nonprofit breast cancer education and support organization located in the Albany/Capital region of New York. Founded by a breast cancer survivor in 1998, our mission is to educate the community about breast cancer detection, treatments, and related health matters and provide support services to breast cancer patients, caregivers, family, and friends. Drawing on To Life! staff's extensive experience in working with the needs of this population, an intervention was designed to communicate to future health care providers information that breast cancer survivors wanted them to know. "Treating the Whole Patient" is an educational intervention designed for students in the health care professions to raise awareness of the psychosocial needs of breast cancer patients and to provide strategies for meeting these needs through the use of available community resources. This project was funded by a grant from the New York State Department of Health's Health Research Science Board.

Description: An initial survey of breast cancer patients was conducted to determine treatment concerns. The results indicated that breast cancer patients have a variety of concerns about the attitudes and behaviors of medical caregivers. Those surveyed offered a number of suggestions for improving the quality of provider interaction. Findings were incorporated into the development of course content. Lecture format presentations with a written course outline and full notes were provided to participants. Presenters generally included at least one breast cancer survivor. Course content was shaped by elicited feedback from audience members and presenters.

Results: Since the first presentation to students in March 2003, over 300 medical, nursing, and physician assistant students have participated in this program. Using pre- and posttests, we were able to assess positive individual-level changes in program participants' knowledge about course content and of resources for addressing high-lighted issues with patients and family members. The project resulted in continuing collaborations between staff of To Life! and Albany Medical College Department of Obstetrics and Gynecology, Columbia Community College School of Nursing, and Albany Hudson Valley Physician Assistant Program.

Conclusions: Relationships forged during the development of this program have resulted in its regular presentation to medical interns, residents, and practicing physicians at grand rounds at Albany Medical Center and at St. Peter's Hospital. Additional presentations, including instruction in breast self-examination technique have been requested on a regular basis by the School of Nursing at Columbia Greene Community College and by the American Medical Women's Association at Albany Medical College. Staff of To Life! continue to provide community education programs on these topics and to gather and distribute information on new and evolving community resources for breast cancer patients to local health care providers and to patients themselves.

This work was supported by the New York State Department of Health.

P5-6: ADVANCE CARE PLANNING DURING CHEMOTHERAPY

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Advance care planning enables women with breast cancer to proactively communicate their end-of-life care preferences. The purpose of this study was to describe the prevalence and predictors of advance care planning documents over time for women with breast cancer undergoing chemotherapy. This study recruited women with breast cancer from ten different community and comprehensive cancer centers located in three states. Women with breast cancer experiencing active disease, were over the age of 21, and were undergoing chemotherapy. Women had to be cognitively intact, English speaking, able to complete telephone interviews. Women under the care of a psychologist or psychiatrist with diagnosed emotional or psychological disorders were excluded. Once women consented, they completed a baseline interview and interviews at 10 and 16 weeks post baseline. A two level Hierarchical linear model was used to examine change within each woman over time and individual characteristics hypothesized to impact advance care planning. Models included age, education, race, marital status, stage of cancer, cancer recurrence, metastasizes, chronic health conditions, optimism, depression, mastery, communication, and trust. Among the 236 women undergoing chemotherapy, the mean age was 53 ± 11 years. Over half of the women were married (n = 151, 64%), had some college education or higher (n = 178, 75%), and reported their racial background as Caucasian/white non-Hispanic (n = 199, 84%). One hundred seventy-seven women (75%) reported having at least one other chronic health condition along with their diagnosis of breast cancer. The majority of women had a diagnosis of late-stage cancer (n = 177, 75%), with 28% (n = 65) of women with breast cancer having had a recurrence in their cancer. One hundred twenty-five (53%) reported having metastatic breast cancer. Seventy-six women (32%) had advance care planning documents. Increased probability of having advance care planning documents was associated with increased age, being White,

and trust in the oncology health care provider. Improving oncology provider trust is a potentially promising mediator of advance care planning document completion among women with breast cancer undergoing chemotherapy.

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P5-7: ESTROGEN RECEPTOR-MAPK CROSS-TALK AS A MECHANISM OF RADIORESISTANCE OF BREAST CANCER

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Since radiation therapy plays a critical role in the management of a majority of breast cancer patients, identification of factors that help predict which patients are at risk for relapse within the irradiated field remains an active area of investigation. A substantial amount of research has been devoted to identifying predictive markers for radiation resistance. Loss of estrogen receptor (ER) function has been associated with constitutive and hyperactive MAPK (particularly ERK1/2), which culminates in aggressive, metastatic, radiation-resistant cancers. Activation of the ERK1/2 cascade modulates the phosphorylation and activity of several nuclear transcription factors that in turn regulate a series of genes involved in promoting cellular survival and resistance to chemotherapy and ionizing radiation. The ERK1/2 pathway has also been linked to DNA damage and DNA repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling. An important hallmark that dictates the radioresistant phenotype of tumor cells and is probably the most critical factor in the radiation responsiveness of a tumor is the ability of a cancer cell to repair and recover from radiation-induced DNA double-strand breaks (DSBs). An increased DNA repair capacity in ER-a negative breast tumors has also been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER-a negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

To test our hypothesis that ER-a negative breast cancer cells are radioresistant due to hyperactive ERK1/2 signaling and high expression of DNA repair proteins, we used ER-a negative (MDA-MB-231 and Hs578t) and ER-a positive (MCF-7 and ZR75-1) human breast cancer cells and compared them for expression levels of ERK1/2 and several DNA repair proteins involved in the repair of radiation-induced double-strand breaks. Radiosensitivity was assessed by clonogenic cell survival assays. Preliminary data obtained from clonogenic cell survival assays showed that ER-a positive cell lines were more radiosensitive compared with the ER-a negative cell lines. These cell lines are also being compared for the expression of ERK1/2 and its downstream proteins and proteins involved in DNA repair by western blot analysis. We are also evaluating the ability of inhibitors of the ERK1/2 pathway to restore radiosensitivity to the ER-a negative cell lines. The effect of these inhibitors on expression of DNA repair proteins and their ability to restore ER-a expression will also be tested. The outcome of these studies will have a potential impact in the clinic and benefit breast cancer patients.

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P5-8: USE OF MITOCHONDRIA-SPECIFIC DYE MKT-077 TO INCREASE PRE-IRRADIATION OXYGEN LEVELS IN EXPERIMENTAL BREAST CANCER

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Background and Objectives: The response of tumors to radiation therapy is dependent upon oxygen levels, and tumors with low oxygen levels (i.e., hypoxic tumors) are radioresistant. Over 60% of human breast tumors are severely hypoxic. Of the two methods to increase oxygen levels, inhibition of tumor cell oxygen consumption is theoretically a better method than increasing the oxygen supply. The rhodacyanine dye analog MKT-077 has been shown to inhibit mitochondrial respiration in some cancer cell types. Based on these considerations, we hypothesized that (1) MKT-077 would be taken up by breast cancer cells and decrease cellular oxygen consumption in vitro and (2) infusion of MKT-077 would decrease oxygen consumption and increase the oxygen levels in a rat model of breast carcinoma. If oxygen levels can be increased, then infusion of MKT-077 before radiotherapy should increase radiation response and permit lower levels of radiation to be used for preoperative treatment of locally advanced breast cancer (LABC).

Methodologies: R3230Ac rat mammary adenocarcinoma cells were grown in vitro while exposed to air (21% O₂) or while exposed to hypoxia (2.5% O₂ or 1% O₂). Cells were harvested and exposed to different concentrations of MKT-077. Drug uptake was determined using spectroscopy, and cellular oxygen consumption was measured using a polarographic electrode in a metabolic chamber. Orthotopic tumors were grown by implanting pieces of R3230Ac tumor into the mammary fat pad of female Fischer 344 rats. When tumors reached 6-8 mm³, rats were anesthetized with pentobarbital, and tumor oxygen tension (PO₂) was measured at a single location

before and after intravenous drug infusion with a polarographic oxygen microelectrode. Blood pressure and blood flow (laser Doppler flowmetry) were also measured.

Results: Cellular uptake of MKT-077 was dose dependent, and the uptake rate was greater for cells grown on air compared to cells grown under hypoxic conditions. Interestingly, exposure of cells grown under hypoxic conditions to air during drug exposure led to a 114%–216% increase in uptake rate compared to the rate if cells remained hypoxic during drug exposure. MKT-077 significantly inhibited oxygen consumption in a dose-dependent fashion, with decreases ranging from 40%–65%. Although extremely effective as an inhibitor in vitro, the effect of the drug in vivo has been more complex. Infusion of 10 mg MKT-077/kg at a rate of 1.25 mg/(kg min) decreased blood pressure and blood flow by 15% and 17%, respectively, and decreased PO₂. Infusion of 7.5 mg MKT-077/kg at a rate of 1.25 mg/(kg min) resulted in no significant change in PO₂ while a slower infusion rate of 0.125 mg/(kg min) appears to increase tumor PO₂.

Conclusions: MKT-077 is a potent metabolic inhibitor of breast carcinoma cells in vitro, but drug delivery must be optimized in order to maximize the in vivo effect. Based on the in vitro data, inhalation of hyperoxic gases during MKT-077 infusion might increase drug uptake by the tumor. If MKT-077 is eventually effective in vivo, its use in combination with radiotherapy could be helpful to patients with LABC and to patients with early stage breast cancer who are receiving radiation as part of breast—conserving treatment or locoregional post-mastectomy radiotherapy. An efficient, safe radiosensitizer could decrease the necessary radiation dose, resulting in less collateral damage to normal tissue and better cosmetic outcome.

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P5-9: ABSTRACT WITHDRAWN

P5-10: PRONE ACCELERATED PARTIAL BREAST IRRADIATION AFTER BREAST-CONSERVING SURGERY: CLINICAL RESULTS

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We report the clinical results of ninety-nine consecutive patients accrued to a post-segmental mastectomy IRB-approved protocol of hypo-fractionated prone partial breast radiation therapy. The intent was to treat the equivalent of a quadrant of breast tissue while sparing the lung, heart and as much remaining breast tissue as feasible.

Materials and Methods: Post-menopausal women with non-palpable T1N0 breast cancer, completely excised with at least 5 mm margins, lack of EIC, and positive hormonal receptors were eligible. All patients were first offered standard six-week radiation treatment and, only upon refusal, were offered the research protocol. After giving an informed consent, the patients accrued to the study underwent a planning CT in the prone position lying on a table where the target breast tissue falls freely through an opening, allowing for isolated targeting of the breast and avoidance of normal structures such as heart and lung. The breast tissue and tumor bed, identified at CT as the post-surgical cavity, were contoured on a 3D planning system (Varian Somavision/CadPlan/Eclipse) and a 1.5 to 2 cm margin was added to create the PTV. The PTV received > 90% of the prescription dose prescribed to the 95% Isodose line. Six Gy per fraction was delivered in 5 fractions to a total dose of 30 Gy over ten days. A 2-stage Simon’s design with projected enrollment of at most 99 patients is used (p=.05; power=.80).

Results: All 99 patients completed treatment. Median age was 68 years (range: 53–88). Median tumor diameter was 0.9 cm (range 0.2–1.9). Planning in the prone position was feasible in 94 of 99 patients. Five patients were treated supine, due to inability to lie comfortably in the prone position secondary to paraplegia, and medial position of the tumor bed. The predominant technique for treatment was a pair of parallel-opposed tangents. This arrangement assured good coverage given the constraints imposed by the PTV and its relationship to the table. We found heterogeneity of DVH based on the position of the original tumor bed and the size of the breast. For the entire group the volume of breast tissue included by the 95% isodose ranged between 13% to 45%. In all patients, volumes of heart and lung included in any of the treatment fields were clinically insignificant. Except for grade 1 erythema in 42 (42%) and grade 2 erythema in 7 (7%) and 1 grade 3 (1%), no other acute toxicity was detected. At a median follow up of 38.6 months (range 2.0–78.7), there are no grade 3 or 4 late toxicities, there was one ipsilateral recurrence and one patient with a contralateral breast cancer. One patient expired from a second primary tumor. Evaluation of cosmesis by MD/NP and by the patient (PT) was performed at 2 years or more follow-up and graded (by MD/NP, by PT) as: excellent (63%, 55%), good (28%, 33%), fair (7%, 9%) and poor (2%, 3%).

Conclusion: Prone partial breast radiotherapy is feasible and, at a median follow up of 38.6 months one ipsilateral recurrence has occurred in this selected series of eligible patients. Cosmetic results were good to excellent for the vast majority of patients.

More follow-up is needed to assess the effectiveness of partial breast irradiation in preventing regional recurrences.

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P5-11: NON-INVASIVE IMAGING TECHNIQUES QUANTITATE RADIATION-INDUCED VASCULAR CHANGES IN THE BREAST

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Purpose/Objectives: Radiation therapy (RT) is a standard modality used to treat breast cancer; however, our ability to individualize treatment is limited by lack of understanding of how radiation effects vary from patient to patient. Recent studies have shown great promise for the use of diffuse optical spectroscopy (DOS) and contrast-enhanced magnetic resonance imaging (cMRI) in measuring physiological changes in breast tissue in response to chemotherapy. The objectives of this study were to determine the feasibility of DOS and cMRI to non-invasively detect radiation dose-related changes in invasive breast cancer (IBC) patients.

DOS/ cMRI Parameters	Scan 1	Scan 2	Scan 3	p-value
THC (μM)	15.8 ± 2.4	22.8 ± 6.8	25.3 ± 7.4	< 0.03
HbO ₂ (μM)	10.7 ± 2.2	16.7 ± 6.0	19.4 ± 6.4	0.02
O ₂ Sat (%)	67.0 ± 5.5	72.4 ± 7.1	76.4 ± 3.3	0.08
PE (%)	13.0 ± 6.0	21.2 ± 7.4	23.9 ± 10.4	0.08

Table 1. Change in DOS/cMRI Parameters with Radiation

Methods: We report our initial findings among eight women who underwent RT following lumpectomy for IBC. Each patient had three serial DOS and cMRI scans of the involved breast: Scan 1 before lumpectomy, Scan 2 after the first week of RT, and Scan 3 after completion of RT. cMRI scans were processed to obtain global peak enhancement (PE) values reflecting the contrast kinetics of non-tumor bearing breast fibroglandular tissue in the involved breast. DOS measurements were obtained for total hemoglobin content (THC), oxyhemoglobin (HbO₂), and oxygen saturation (O₂Sat). Measurements were taken at the same point for each scan, at least 5 cm away from the tumor bed. DOS parameters and PE were analyzed using the Wilcoxon chi-square test for statistical significance.

Results: Mean age of the cohort is 48.5 (range 36–60). Among the DOS parameters, THC (p<0.03) and HbO₂ (p=0.02) increased significantly from Scan 1 to Scan 3. In addition, there was a trend toward increased O₂Sat (p=0.08), Table 1. The most significant changes occurred with the onset of RT and persisted post-RT. An increasing trend in global PE (p=0.08) from cMRI analyses was also observed, Table 1.

Conclusions: Measurements taken from eight women with IBC show that DOS parameters (THC and HbO₂) increase with RT, and PE values from cMRI parallel these changes. These physiologic measurements reflect global changes in breast vascularity associated with RT. These results indicate that DOS and cMRI measurements have good reproducibility for individual patients and may be promising tools to characterize dose-dependent radiation changes in the breast.

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P5-12: AN “IN VITRO CELL-BASED SYSTEM” TO ASSESS RADIATION SENSITIVITY IN SPORADIC BREAST CANCERS

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Breast cancer is the second leading cause of death in women. Treatment for breast cancer usually involves lumpectomy followed by a regimen of radiation, chemotherapeutic and hormonal therapies. Radiation therapy is widely used to prevent metastases from forming; however, it is difficult to predict how epithelial cells and tumors respond to radiation treatment. To help predict response to X-ray treatment and aid in the development of agents that sensitize breast cancer to X-ray treatment, we are developing an “in vitro cell-based system” to assay X-ray sensitivity from a subset of breast cancer cell lines originally derived from sporadic breast tumors. We have performed colony formation assays following X-ray treatment and observed that some breast cancer cell lines are quite sensitive to X-ray treatment (HBL100 and ZR75-1) while others are quite radio-resistant (MCF7 and MDAMB231). Many inherent factors may modulate cellular response to DNA damage, examples being the levels and activities of tp53 or Bcl-2 family members. How-

ever, none of these factors in our study or others have proven themselves to be truly accurate predictors for radio-sensitivity/resistance in breast cancer.

Since the non-homologous end-joining (NHEJ) pathway is a major determinant of DNA repair and cellular survival after X-ray treatment, we are interrogating members of this pathway in breast cancer and normal human mammary epithelial cells in response to X-ray treatment. Specifically, we are studying the roles of the *DNA-dependent protein kinase (PRKDC)* and the newly discovered NHEJ gene, *Artemis (DCLRE1C)*, in predicting the responses of breast cancer cells to X-ray treatment. Using RNAi technology and lentiviral systems, we are inhibiting these members of the NHEJ pathway to modulate radiation sensitivity in breast cancer. Our studies show that Artemis steady-state protein levels are elevated in some of the more radio-resistant breast cancer cell lines compared to normal human mammary epithelial cells and radio-sensitive breast cancer lines. We have also sensitized several of the most radio-resistant breast cancer lines to X-ray treatment by reducing Artemis protein levels using RNAi-based technologies. These data suggest that Artemis expression may act as a predictor for radio-therapy and more importantly that it is a potential therapeutic target to increase the efficacy of radiation usage for breast cancer treatment. To validate our "in vitro cell-based system," we will be performing mouse xenograph assays to sensitize the most radio-resistant breast cancer cell lines by shRNA and siRNAs directed at *Artemis* and *PRKDC* using neutral liposome-mediated technologies. We will also be using our newly developed "in vitro cell based system" to help identify other novel predictors and intervention targets for radio-therapy in breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0527.

P5-13: FEASIBILITY OF A COMBINED KYPHOPLASTY AND BRACHYTHERAPY PROCEDURE FOR VERTEBRAL METASTASES

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Introduction: Spinal metastases are a common and serious manifestation of breast cancer. These lesions can be extremely painful and can lead to vertebral collapse and neurologic complications. Treatment often involves a surgical procedure—kyphoplasty or vertebroplasty—that involves injecting bone cement (polymethyl-methacrylate) into the vertebral body to stabilize the region, followed by external beam radiation therapy (EBRT) to control tumor growth. To address the limitations of EBRT (e.g., risk to the spinal cord), we propose to combine kyphoplasty or vertebroplasty with radiotherapy by mixing a radionuclide with the injected cement, thereby providing structural reinforcement to the bone while simultaneously irradiating the tumor from within (i.e., spinal brachytherapy). This study evaluated the feasibility of using this radioactive bone cement for delivering therapeutic radiation to the vertebral body while sparing the spinal cord.

Methods: Monte Carlo radiation transport modeling (MCNPX v.2.5.0, Los Alamos National Laboratory) was performed using a CT scan-based model of a T-6 vertebra containing a 1.27 cm-diameter \times 1.5 cm-height cylindrical volume of radioactive bone cement. The CT scan data were transformed into a three-dimensional rectangular lattice of 0.32 mm \times 0.32 mm \times 3 mm (CT slice thickness) voxels. Each voxel was assigned one of nine material definitions: seven representing bone of various volume fractions and compositions of marrow and solid bone (1.9 g/cm³),¹ with densities calculated from the CT data; one representing all soft tissue (spinal cord, muscle, etc.) with constant composition and density (1.0 g/cm³);¹ and one representing Surgical Simplex® P Radiopaque Bone Cement (Stryker, Kalamazoo, MI). Two candidate radionuclides, P-32 and Sr-89, each previously FDA approved for treatment of bone metastases, were modeled as uniformly distributed sources within the cement. Thirty million particle histories were simulated to determine the dose deposited in voxels surrounding the cement, and the results were linearly scaled to determine the activity required to deliver the maximum possible dose to bone while keeping the spinal cord dose under 45 Gy (maximum tolerable).

Results: The dose distributions for both radioisotopes showed similar characteristics, demonstrating axisymmetric distributions about the cement implant and rapidly decreasing dose with increasing distance from the cement. Activities of 0.93 mCi and 0.51 mCi for P-32 and Sr-89, respectively, delivered over 130 Gy to bone within 1.8 mm of the cement implant and over 60 Gy to bone within 2.6 mm while keeping the dose to the spinal canal, located 2.9 mm from the cement implant, under 45 Gy.

Discussion: The predicted dose distribution would deliver a therapeutic dose to much of the vertebral body without undue risk to the spinal cord, indicating potential feasibility of this technique. Future development will be directed at achieving a more complete therapeutic result by refining the choice of radioisotope(s), amount of activity, and geographic distribution of the cement. If successful, this procedure would eliminate the need for about 10 radiation therapy sessions, making it convenient for the patient, would allow for a higher dose to the bone metastases, potentially improv-

ing clinical outcome, and would lead to a lower dose to the spinal cord and other normal tissues.

Reference:

1. Kramer R et al. 2006. *Phys. Med. Biol.* 51:3331-46.

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P5-14: PILOT STUDY OF HORSE THERAPY: AEROBIC CAPACITY AND QUALITY OF LIFE OF BREAST CANCER SURVIVORS

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Background: No research has examined the effect of an Equine Assisted Growth and Learning horse therapy (HT) program on breast cancer survivors. Observation suggests that breast cancer survivors who engage in a HT program gain physical endurance, improve confidence and self-esteem, and feel better emotionally. While the body of research on exercise and physical activity for cancer survivors has greatly expanded and is moving toward becoming standard of care, there is no information on the physical and emotional effects of HT on breast cancer survivors. This study will examine the effect of HT on cancer survivor's aerobic capacity and quality of life. It is hypothesized that HT will improve both aerobic capacity and quality of life.

Purpose: This pilot study is a randomized cross-over trial examining the effects of an Equine Assisted Growth and Learning HT program on the quality of life of breast cancer survivors.

Methods: Subjects (N=20) breast cancer survivors who are beginning a HT program will be invited to participate and randomly assigned to the HT intervention or wait-list control. Wait-list control subjects will begin the HT intervention after the first group has completed the eighth session. The initial intervention group will then act as the control group. Consent will be obtained, subjects will be randomized to group, and then complete baseline measures (6-minute walk test, Positive and Negative Affect Scale state and trait (baseline only), Side Effect Symptom Checklist, Schwartz Cancer Fatigue Scale (SCFS), and SF-36) and be asked questions about their medical history. The 6-minute walk test will ask subjects to walk or run as far and as fast as they can for 6 minutes. They can slow down and rest as needed. This test will be performed over level ground following a pre-measured course. Subjects will complete the questionnaires after the fourth session, and both the questionnaires and 6-minute walk after the eighth session.

Results: The study is ongoing. Eight subjects have been enrolled, and no data analysis has been complete at this time. The completed analysis will include descriptive analysis and analysis of variance to group differences on measures quality of life, and an examination of the long-term effect of HT on the first intervention group. Adherence to the program will also be reported.

Conclusions: Results of this pilot study may provide information on the effect of HT on breast cancer survivors and may eventually become another mode of physical and emotional therapy for cancer survivors.

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P5-15: REGRESSION ON MEDIAN RESIDUAL LIFE

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A time-specific log-linear regression method on the median residual lifetimes is developed. Under the new regression model, the median of a time-to-event distribution among survivors beyond a certain time point is associated with selected covariates under right censoring. It is noted that an estimating equation for the regression coefficients can be still formulated on the original scale of observed survival times. Consistency and asymptotic normality of the regression parameter estimator are established. An asymptotic test statistic is proposed to evaluate the covariate effects on the median residual lifetimes at a specific time point. Evaluation of the test statistic does not require estimation of the variance-covariance matrix of the estimates of regression coefficients. Simulation studies are performed to assess finite sample properties of the regression parameter estimator and test statistic. The new regression method is applied to a breast cancer data set with more than a quarter century of follow-up from National Surgical Adjuvant Breast and Bowel Project to estimate the pattern of the patients' median residual lifetimes adjusted for important prognostic factors in breast cancer as time progresses. These estimates may be useful as baseline mortality information for designing clinical trials on breast cancer where a new intervention is expected to be given to patients in the middle of the follow-up period.

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P5-16: POLYMER BASED ADJUVANT THERAPY FOR TREATMENT OF BREAST CANCER

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Early breast cancer may be treated with a combination of breast-conserving surgery plus adjuvant breast irradiation. A goal of radiation is to minimize outgrowth of residual tumor. Radiation increases the cosmetic deformity of surgery (Figure 1). Polymer/chemotherapy conjugates have been developed for regional therapy of brain metastases. A similar approach could serve as an effective adjuvant option for patients undergoing breast conservation surgery for breast cancer. We report a preliminary assessment in a murine model of a biodegradable polymer bound chemotherapy release device that effectively controlled local tumor growth while simultaneously acting as a soft tissue support. Poly(lactic-co-glycolic acid) microspheres were used to encapsulate Doxorubicin. Microspheres were embedded in gelatin scaffolds generating a biphasic construct. Release and degradation kinetics of the construct are tailored according to scaffold design. Bioactivity and drug release kinetics were determined in a series of in vitro experiments (see figure 2). 4T1 murine mammary cancer cells were injected into the mammary fat pad of female BALB/c mice. Constructs containing drug loaded or empty polymer microspheres were then injected at the time of tumor inoculation. Tumor size was recorded and animals imaged with x-ray. The implanted construct was completely radiotransparent. The drug containing construct effectively controlled tumor outgrowth ($P < 0.05$) without tissue toxicity.



Figure 1

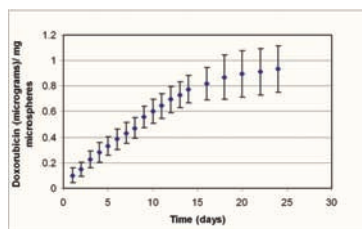


Figure 2

This study suggests that local delivery of anticancer drugs by polymeric microspheres embedded in biodegradable scaffolds may be an effective treatment adjunct for patients with breast cancer and may improve the aesthetics for women undergoing multimodality therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0725.

P5-17: AUTOMATED CANCER CELL SURGICAL MARGIN DETECTOR

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Breast conservation therapy (BCT) is the primary treatment option for early stages of breast cancer, but 20%–40% of these patients will need a second surgery due to the lack of a practical intra-operative margin evaluation technique. Our group is developing an automated system that intra-operatively analyzes the margins of the removed tumor via touch prep samples to assure the absence of cancer cells in the excised tissue. We tested poly-L-lysine coated slides on 12 human surgical samples including tumor and normal human breast tissue coming from BCT and breast reduction surgeries. The yield of total cells and epithelial cells was 8x greater from tumor samples than normal tissue. The density of total cells was 227.8 ± 161.3 versus 40.8 ± 9.1 ($p=0.15$) cells on average per mm^2 while the total number of epithelial cells was 102.8 ± 127.7 versus 13.6 ± 11.8 cells on average per mm^2 . While the number of epithelial cells alone is a strong indicator of cancer, to improve the selectivity/specificity of cancer cell detection, automated microscopy is being employed to rapidly identify cancer cells. We employed known cancer and benign cases as a training set. Hematoxylin and eosin (H&E) stained histological sections were analyzed for nuclear features using an automated nuclear analysis system, which can outline and extract measurement parameters both from individually isolated nuclei and from groups of cells. The isolated characteristics included area, circularity, Feret's diameter, maximum, mean, minimum, standard deviation of staining intensity and perimeter. The group characteristics included the minimum local inter-nuclear distance, the local nuclear density, the fractional nuclear area, fraction of area covered by high circularity nuclei, and fraction of nuclei with high circularity. The surgical samples were classified into high-grade ductal carcinoma in situ (HGDCIS), low-grade ductal carcinoma in situ (LGDCIS), high-grade invasive (HGINV), low-grade invasive (LGINV), and benign. A total of 25 surgical cases and 25,000+ cells were evaluated. The images

were reviewed by a pathologist to confirm their correct histological designation. Enhanced separation of cancer classes with minimal overlap was achieved. With linear discriminant analysis, 4 variables accounted for 55.92%, 31.12%, 12.04%, and 0.92% of the proportion of discrimination, respectively. The first variable (LDA1) had means of -11.12, -9.83, -9.26, -8.44, and -5.22 and standard deviations of 1.30, 0.51, 1.38, 0.46, and 0.48 for HGDCIS, LGDCIS, HGINV, LGINV, and benign, respectively. Inter-group separation is readily achievable with the first variable alone. For example, for even LGINV and normal cells, the LDA1, LDA2 centroid separation was 6x the standard deviation of the distribution of LDA1, LDA2 parameters; this allows for 99% certainty in discriminating between LGDCIS and normal cells. The enhanced ability to discriminate using automated analysis over manual techniques is to provide fast, accurate, quantitative, and objective data from the inclusion of individual and cell group parameters. The automated cell discrimination technique is presently being tested on malignant and benign tumor margin samples. By combining selective capture of cancer cells with automated analysis, we expect to provide a practical rapid intraoperative method of quantitative pathological analysis for breast cancer surgical margins.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0522.

P5-18: ABSTRACT WITHDRAWN

P5-19: CHEMOTHERAPY INDUCES COGNITIVE DYSFUNCTION IN RATS

Gregory Konat and Jame Abraham

West Virginia University

Although cognitive dysfunction manifested by severe memory and attention deficits has been reported in 20%–30% of breast cancer patients undergoing chemotherapy, the mechanisms of this serious side effect have not been defined. Moreover, it has not been decisively resolved whether the dysfunction is a *bona fide* effect of chemotherapy or is instigated by the malignancy itself. In the present study we tested whether cognitive dysfunction can be induced in an experimental setting by the administration of commonly used chemotherapeutics. Female ten month old (retired breeders) Sprague-Dawley rats were injected intraperitoneally with a combination of 2.5 mg/kg of adriamycin (A) and 25 mg/kg of cytoxan (C). A total of four doses were given at weekly intervals. The control group was treated with saline only. No apparent morbidity was observed in either group. However, the AC-treatment severely impaired memory function of rats as measured by passive avoidance test. This memory deficiency was fully prevented by 200 mg/kg of N-acetyl cysteine (NAC) injected subcutaneously three times a week in the course of AC-treatment. Our experimental paradigm provides a convenient model system to study the mechanisms of brain damage elicited by chemotherapy, and for testing the potency of putative preventive agents. Moreover, our results indicate that NAC has a great potency to ameliorate chemotherapeutic agent-induced cognitive dysfunction.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0620.

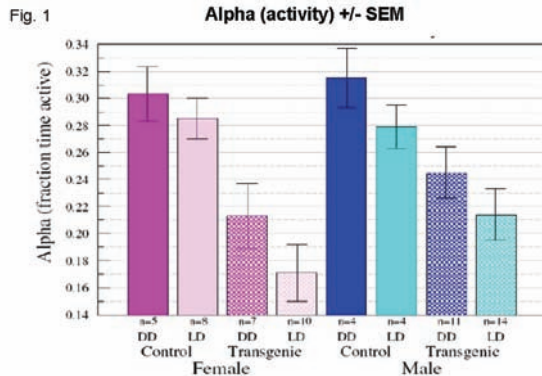
P5-20: THE TRANSGENIC TGF- α OR EGFR1 OVEREXPRESSION MOUSE MODEL FOR SYMPTOM COMPLEX RESEARCH

Tyvin Rich, Meredyth Evans, Rohan Patel, and Sarah Parsons

University of Virginia

Background: Fatigue, appetite loss, and sleep disruption are common in breast cancer patients. This symptom clustering suggests a shared biologic mechanism that may be explained by the hypothesis based on *targeted neural signaling* by transforming growth factor- α (TGF- α), a ligand of the epidermal growth factor receptor-1 (EGFR1), since TGF- α acts to inhibit EGFR1 expressing hypothalamic modulatory centers that regulate 24-hour rest/activity, core body temperature, and food intake patterns. Human data supporting this model demonstrate a significant relationship exists between pre-treatment serum levels of TGF- α and high levels of fatigue, appetite loss, and abnormal 24-hour rest/activity patterns in at least one cancer population. Another relevant clinical observation is that EGFR1 inhibition has been associated with rapid improvement of quality of life indices in cancer patients. These limited clinical data support further evaluation of the EGFR ligand hypothesis as a model for symptom production in breast cancer patients.

Methods: We have used a transgenic C57 BL/6J mouse harboring an MMTV promoter-driven human TGF- α and measured their 24-hour rest/activity compared to those obtained from nontransgenic normal controls. After genotyping, 24-hour rest/activity patterns (actigraphy) were measured continuously in 10- to 14-week old transgenic mice for 3 weeks with computerized running wheels housed in a light/temperature-controlled environment; conditions included 12-hour light/dark (LD) and constant darkness (DD).



Conclusion: Controls exhibit more relative active time than do transgenics.

Alpha is a measure of the relative duration active (a value of zero meaning no activity, a value of unity corresponding to no rest).

p-value 2-sided < 0.0001 (Welch's t-test used because variances not equivalent; p(SD) = 0.0011)

Results: Running wheel behavior for the TGF- α overexpression mice compared to the controls reveals no statistical difference between the phase of the running wheel behavior in either LL or LD conditions for either gender. In contrast, there is evidence for significantly decreased activity in the transgenic TGF- α mice compared to controls (Figure 1) and this is evident in either LD or DD conditions.

Comment: Our initial studies are consistent with the EGFR-ligand hypothesis that predicts actigraphy in transgenic TGF- α overexpressing mice is suppressed. Since there is no effect on the central pacemaker (suprachiasmatic nucleus) based on the lack of phase differences of behavior, the target for fatigue (activity loss) in the transgenic mice may be downstream in EGFR expressing nuclei of the adjacent hypothalamus. Data will be presented with the use of specific EGFR tyrosine kinase inhibitors (AG1478 and TARCEVA) used to reverse the effects of TGF- α on fatigue in this model.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0666.

P5-21: THE ROLE OF SEROTONIN IN HOT FLASHES AFTER BREAST CANCER

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Background: Among women with breast cancer, a hot flash is a frequent, severe, and bothersome symptom that is negatively related to mood, affect, and daily activities

and can compromise compliance with life-saving medications (e.g., tamoxifen). Over 60% of breast cancer survivors report hot flashes with 59% stating they are extremely severe and 44% reporting them to be extremely bothersome. Unfortunately, poor understanding of hot flash physiology limits clinicians' abilities to fully treat this symptom. Although the current nonhormonal treatment of choice for hot flashes after breast cancer targets the central serotonin system (e.g., paroxetine and venlafaxine), the role of serotonin in hot flashes has not been directly tested. Because the effectiveness of serotonergic agents has been based largely on improvement in subjective reporting of hot flashes, it is not clear whether benefits are due to physiological effects on hot flashes or due to improvements in mood or other related symptoms. In addition, these and other currently available treatments are not acceptable, appropriate, or effective for all women with breast cancer. Understanding the physiological mechanisms involved in hot flashes after breast cancer will enable us to develop more targeted behavioral and/or pharmacological therapies to be used in lieu of, or in addition to, currently available therapies so that we can eradicate hot flashes and improve the quality of life for women with breast cancer.

Objective/Hypothesis: The study purpose was to understand the role of serotonin in hot flashes by altering central serotonin concentrations using a well-established acute tryptophan depletion paradigm. The main hypothesis was that alterations in central serotonin levels are involved in the induction of hot flashes in women with breast cancer and that variability in response to serotonin manipulation can be partly explained by genetic variations in serotonin receptors and transporters.

Study Design: A within subjects, double-blind, placebo-controlled, balanced, cross-over study design was used. After fasting overnight, each participant took part in two similar 9-hour test days within the General Clinical Research Center. On one test day, women with breast cancer ingested a concentrated amino acid drink and encapsulated amino acids (no tryptophan) according to published procedures that have been previously shown to have specific effects on serotonin within 4.5 to 7 hours. On the other test day, women ingested a ¼ strength amino acid drink that was identical in taste, color, and volume and was previously shown to have no effects on serotonin. Women were assessed the day after each test day for adverse effects. Serial venous blood sampling was used throughout each test day to monitor response to each condition. Hot flashes were monitored using objective sternal skin conductance monitoring.

Relevance: Results will help guide the development of improved interventions for alleviating hot flashes in women with breast cancer. If findings are positive, future interventions may target the central serotonin system either behaviorally (e.g., diet) or pharmacologically (e.g., alternative drug therapeutics). If findings are negative, they will be equally as useful in guiding future research on nonserotonin-related etiologies and interventions.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0326; Indiana University General Clinical Research Center; and National Institutes of Health (M01 RR00750).

COMPLEMENTARY AND ALTERNATIVE MEDICINE

Poster Session P6

P6-1: ESTROGENICITY OF A BROAD SPECTRUM OF MEDICINAL BOTANICAL EXTRACTS

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In recent years, use of complementary and alternative medicines has increased among both healthy individuals and cancer patients. Extracts derived from medicinal plants have been used for gynecological complaints, as immune modulators and adjuvants to chemotherapy, and to treat various chronic illnesses. The popularity of these medicinal botanicals (MBs) may be related to their perceived safety and accessibility for self-treatment. However, these products may pose a particular problem for persons with, or at risk for, hormone-responsive cancers since safety and efficacy claims for these extracts rarely have been verified, and little is known about their hormonal properties. Our previous work has determined that extracts of several plants traditionally used for gynecological complaints interact with estrogen receptor (ER) in vitro and induce estrogenic responses in ovariectomized female rats. Our aim in these studies was to investigate the estrogenicity of MBs used as immune modulators and general tonics, as well as for other purposes. Estrogenicity of MB extracts was evaluated in vitro using competitive ER α and ER β binding assays and a cell proliferation assay (CPA) utilizing human breast cancer cell lines MCF-7 (mainly ER α -positive), T47D (mainly ER β -positive), and BT20 (ER-negative) in the presence and absence of a physiological level of estradiol (E2, 1nM). The MBs tested were standard extracts of saw palmetto (SP), red clover (RC), dang gui (DG, angelica); milk thistle (MT), Huang Qi (HQ, astragalus); gang cao, (GC, licorice), ling zhi (LZ, reishi mushroom), he shou wu, (HSW, fo-ti). Eight additional extracts were also tested in ER α binding assay. Significant dose-dependent inhibition of radiolabeled estradiol binding to ER α was observed with the most potent extracts being SP, RC, DG, GC, and HQ. LZ exhibited moderate competition while HSW exhibited slight competition in this assay. In contrast, estradiol binding to ER α was enhanced by extracts of MT. Significant dose-dependent inhibition of radiolabeled estradiol binding to ER β was also observed with the most potent extracts being SP, RC, DG, and GC. HQ and HSW exhibited moderate competition while LZ exhibited slight competition in this assay. Again, in contrast, estradiol binding to ER β was enhanced by MT. In the CPA, effects of the various herbal extracts differed with dose and presence of E2. The ER-negative BT-20 cells exhibited no growth stimulation when treated with any of the herbs or with E2. In MCF-7, RC, GC, and HQ had strong proliferative effects; SP, DG, LZ, SP, and MT were modestly proliferative; and HSW had no effect. In contrast, in T47D, RC, SP, HQ, DG, LZ, and GC had strong proliferative effects, and MT and HSW were modestly proliferative. In general, T47D cells are better responders to these MBs, most likely because of their higher content of ER β , which has a higher affinity than ER α for phytoestrogens. In conclusion, these botanicals demonstrate varying degrees of estrogenicity. They interact with both ER α and ER β , and in the presence of a physiological E2 level, may amplify ER-mediated cellular events. Both cell lines demonstrate proliferation in the presence of these extracts with T47D showing a greater response. While these botanicals may have clinical applications, they should be avoided or used cautiously by patients with hormone-responsive diseases.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0436 and Veterans Research Foundation of Pittsburgh.

P6-2: INVESTIGATION OF INFLAMMATORY BREAST CANCER BIOLOGY AND POTENTIAL THERAPEUTIC APPROACHES

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¹University of Texas at Austin and ²Universidad Central Del Caribe

Inflammatory breast cancer (IBC), the most lethal and least understood form of locally advanced breast cancer, manifests itself with inflammatory-like symptoms and is associated with increased vasculogenesis and local invasion of the lymphatic system. We used SUM-149, a human inflammatory breast cancer cell line, to characterize the unique IBC phenotype in vitro. Results showed that IBC cells use a different mode of invasion from other metastatic breast cancer cells and invade as tumor spheroids in a Matrigel matrix, similar to the tumor emboli seen in IBC pathology. As reported previously by van Golan et al., the IBC cells over expressed Rho and demonstrated more actin stress fibers. IBC cells also overexpressed E-cadherin and retained E-cadherin-based cell-cell adhesions in the tumor spheroids during invasion in 3-D Matrigel cultures (Hoffmeyer et al., 2005. *Cancer Cell Int.* 5:11). Current therapies for IBC include aggressive nonspecific anticancer treatments such as paclitaxel and doxorubicin that have drastic side-effects. To investigate the role of alternative targeted therapeutics that can be used in combination with current therapeutic options we are determining the efficacy of *Ganoderma lucidum* (Reishi) mushroom extract. Reishi mushroom is a traditional Chinese medicinal herb that has been shown to inhibit proliferation, adhesion, migration, and invasion of cancer cells. Reishi possesses biologically active compounds with polysaccharides that stimulate the immune system and triterpenes that demonstrate cytotoxicity against cancer cells at high concentrations. To test the hypothesis that the immunomodulatory, anti-inflammatory, and anti-cancer

effects of Reishi may be effective against IBC progression and invasion, we tested the effect of whole Reishi extract on normal mammary epithelial (MCF-10A) and IBC (SUM-149) cell lines. Treatment with Reishi extract effectively inhibited proliferation of the IBC cell line SUM-149 but not the normal mammary epithelial cell line MCF10A. Vehicle-treated SUM-149 cells invaded a Matrigel matrix as tumor cell spheroids. Reishi treatment reduced cell-cell attachments and decreased invasion of IBC cells. Investigation of gene expression in response to Reishi using RT² profiler cancer pathway finder PCR arrays indicated that a total of 52% of tumorigenesis genes were down-regulated in IBC cells treated with the Reishi extract compared to those exposed to vehicle alone, including matrix-metalloproteinase-9 (MMP-9). Since MMPs are important for degradation of the extracellular matrix, we further investigated the effect of Reishi on MMP levels by gel zymography. Reishi at 0.5 mg/ml inhibited MMP-2 and MMP-9 levels compared to vehicle control. We conclude that Reishi inhibits IBC progression by reducing cell proliferation, preventing the formation of tumor emboli, and inhibiting invasion by reduced matrix MMP levels. Overall, these results demonstrate that Reishi extract is effective in inhibiting IBC progression and is a potential natural therapeutic for women suffering with this deadly disease.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0391 and National Institutes of Health (S06 GM050695 & 2G12RR003035).

P6-3: SELF-HYPNOTIC RELAXATION FOR LARGE CORE BREAST BIOPSY: A PROSPECTIVE RANDOMIZED TRIAL

Elvira Lang, Kevin S. Berbaum, Eleanor Laser, Michael L. Berbaum, and Janet Baum

Beth Israel Deaconess Medical Center

Background & Objective: Large core needle biopsy of suspicious breast findings is typically performed in outpatient settings with local anesthetic only which reduces cost but limits the use of intravenous drugs to reduce pain and anxiety. Procedural distress not only interferes with the procedure at hand but can also have deleterious consequences for subsequent health behavior. Nonpharmacologic adjuncts in these busy settings are largely under-investigated leaving the patients with little or no anxiolytic relief. We therefore assessed whether adjunct self-hypnotic relaxation could provide the patients comfort in a resource sensitive manner.

Methods: 236 women referred for large core needle breast biopsy to an urban tertiary university-affiliated medical center were prospectively randomized to receive standard care (n=76), structured empathic attention (n=82), or self-hypnotic relaxation including structured empathic attention (n=78) during their procedures. The structured empathic behaviors were displayed by an additional team member and consisted of matching the patient's verbal and nonverbal communication pattern, listening attentively, providing the perception of control ("Let us know at any time what we can do for you"), swiftly responding to patient's requests, encouraging the patient, avoiding negatively valued language (e.g., "You will feel a burn and a sting"), and use of emotionally neutral descriptors instead ("This is the local anesthetic"). In the Hypnosis condition, this person also read a standardized hypnotic induction script, and, as needed, addressed the patient's anxiety, pain, or worries according to the prescriptions of the script. All patients received local anesthetic. Patients' self-ratings of pain and anxiety at 10 minute-intervals on 0-10 verbal analog scales with 0=no pain/anxiety at all, 10=worst pain/anxiety possible, were compared in an ordinal logistic regression model. Room time and cost were assessed by univariate ANOVA. Differences in the proportions of adverse events in the 3 groups were evaluated by Fisher exact tests.

Results: Women's anxiety increased significantly in the standard group (logit slope = 0.18, p < 0.001), did not change in the empathy group (slope = -0.04, p = 0.45), and decreased significantly in the hypnosis group (slope = -0.27, p < 0.001). Pain increased in all three groups (logit slopes: standard care = 0.53, empathy = 0.37, hypnosis = 0.34; all p < 0.001) though less steeply with hypnosis and empathy than standard care (p = 0.024 and p = 0.018, respectively). Room time and cost were not significantly different despite hypnosis and empathy requiring an additional professional: 46 minutes/\$161 for standard care, 43 minutes/\$163 for empathy, and 39 minutes/\$152 for hypnosis. There were 7 adverse events in the Standard group (5 hematomas, one vasovagal and one vomiting episode), 11 in the Empathy group (9 hematomas, 2 vasovagal episodes), and 3 hematomas in the Hypnosis group. The proportions of adverse events among groups were not statistically different although the low overall adverse event rate reduced the power of the test.

Conclusion: We conclude that, while both structured empathy and hypnosis decrease procedural pain and anxiety, hypnosis provides more powerful anxiety relief with an excellent safety profile and without undue cost. Offering patients self-hypnotic relaxation during large core breast biopsy appears to be an effective adjunct for management of procedural distress.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0153 and National Institutes of Health, National Center for Complementary and Alternative Medicine (1K24 AT 01074 and R01-AT-0002-07).

P6-4: ELECTROACUPUNCTURE ATTENUATES BONE CANCER PAIN AND INHIBITS SPINAL INTERLEUKIN-1 β EXPRESSION IN A RAT MODEL

Lixing Lao,¹ Ruixin Zhang,² Aihui Li,² Bing Liu,² Linbo Wang,³ Yi Wang,⁴ Ke Ren,³ Jian-Tian Qiao,² and Brian M. Berman¹

¹University of Maryland School of Medicine, ²Dana-Farber Cancer Institute, ³University of Maryland, Baltimore, and ⁴Shanghai University of Traditional Chinese Medicine, Yueyang Affiliated Hospital, Shanghai, China

Background: Although cancer pain affects the quality of life of cancer patients, current medical treatments are either ineffective or have side effects. Using a rat model of bone cancer pain, the present study investigated effects of electroacupuncture (EA) on cancer-induced hyperalgesia and the expression of interleukin-1 β (IL-1 β), the up-regulation of which is related to the maintenance of persistent pain.

Method: Cancer was induced by injecting AT-3.1 prostate cancer cells into the tibia of the male Copenhagen rat. Sham cancer rats received a vehicle inoculation of the tibia. The rats were treated with 10 Hz/ 2 mA/ 0.4 ms pulse EA for 30 min daily at the human equivalent of acupoint GB30 (Huantiao) on days 14–18 after the inoculation. For sham control, EA needles were inserted into GB30 without stimulation. In separate rats, IL-1 receptor antagonist (IL-1ra, 0.1 mg/rat) was intrathecally administered daily between days 14 and 18 to determine the role of IL-1 β in cancer pain. Thermal hyperalgesia, a decrease in paw withdrawal latency (PWL) to a noxious thermal stimulus, was measured at baseline, 20 min after EA treatment, and 2 hr after IL-1ra application on days 15 and 18 post-inoculation. After behavioral testing on day 18, IL-1 β and its mRNA were respectively determined in the spinal cord by immunohistochemistry and reverse transcription-polymerase chain reaction analysis.

Results: Thermal hyperalgesia developed in the ipsilateral hind paw between days 12 and 18 after cancer cell inoculation. EA significantly ($P < 0.05$) attenuated this hyperalgesia, increasing PWL from 7.0 ± 0.3 sec to 9.2 ± 0.4 sec on day 18. The bone cancer also significantly induced an increase of IL-1 β -immunoreactive cells in laminae I-II (49.2 ± 1.2 versus 34.4 ± 2.6 cells per 30 μ m section) and V-VI (74.6 ± 3.8 versus 34 ± 2.2) and an upregulation of its mRNA (232 ± 25 versus 100 ± 0) in the ipsilateral spinal cord compared to the sham cancer. EA significantly ($P < 0.05$) inhibited the up-regulation of IL-1 β (59.0 ± 2.8) and its mRNA (113 ± 5.0) in spinal laminae V-VI. Intrathecal injection of IL-1ra also significantly inhibited cancer-induced thermal hyperalgesia, increasing PWL from 7.4 ± 0.6 sec to 9.9 ± 0.5 sec on day 18.

Conclusion: The data suggest that EA alleviates bone cancer pain at least in part by suppressing IL-1 β expression. The results support the clinical use of EA in the treatment of cancer pain.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9274 and National Institutes of Health (R21 CA102383 and AT002605).

P6-5: ABSTRACT WITHDRAWN

P6-6: ABSTRACT WITHDRAWN

P6-7: PROVIDING INFORMATION ON THE EVIDENCE BEHIND COMPLEMENTARY, ALTERNATIVE (CAM) APPROACHES TO CANCER

Ann E. Fonfa

Annie Appleseed Project

Many studies have shown the strong interest among the patient population for information on complementary and alternative modalities. (Eisenberg et al, 2000; Kumar, 2002; Metz, 2002; Kelly et al, 2002; Richardson et al, 2000; Bernstein & Grasso, 2001; Ashikaga et al, 2002; Wolsko et al, 2004; Risberg et al, 2004; Buettner C et al, 2006; Mansky PJ et al, 2006; McEachrane-Gross FP et al, 2006; Swarup AB et al, 2006; Robotin MC et al, 2006; Humpel N et al, 2006, etc.). CAM is in use everywhere.

But those same studies have indicated that people with cancer do not tell their practitioners about their CAM use unless asked. And most oncology professionals lack the necessary information to hold a meaningful conversation. We decided many years ago that our advocacy at www.annieappleseedproject.org would try to bridge this gap.

Thus, the idea of our conference “Evidence-Based Complementary and Alternative Therapies for Cancer Advocates, Patients, and Interested Others” was born.

In January 2008, The Annie Appleseed Project held its first national meeting. In attendance were patient advocates, patients, family/caregivers, doctors, nurses, nutritionists, other people who work in cancer centers, CAM practitioners, and interested others.

This meeting took a year to plan and execute. Almost 200 people attended with the vast majority from the breast cancer community. Participants were treated to 22 presenters speaking on topics ranging from “Chinese Herbs for Breast Cancer,” “Kitchen

Chemotherapy,” and “Stages of Cancer and Homeopathy,” to “Reiki Vibrational Healing: How It Can Support Your Healing Journey,” “Differences & Similarities between CAM and Conventional Treatment for Research Purposes,” and “Inside the DNA of Cancer Cells and How to Normalize Them,” along with much more. Six patient advocates spoke of their own (or a family members) use of CAM.

The Annie Appleseed Project was able to pay for hotel rooms for the speakers, and in one or two cases of need, their airfare. All donated their time and some their travel costs to present this valuable information.

Evaluations from the conference will be presented at the Era of Hope meeting with quotes directly from the patients and patient advocates who participated. We'll show our conference journal containing the agenda, speakers' bios, and content material.

More than 70 people were given scholarships for hotel rooms (2 nights) and free entry, and an additional 30 participated in the meeting with the entry fee waived. Our goal was to be very inclusive.

During the meeting there were plenty of opportunities to network and exchange ideas—an important facet of this educational conference.

The meeting was audiotaped and because of a grant to a local Florida-based nonprofit, we were able to have the meeting videotaped too.

Future plans include wide distribution of CDs and videotape, producing a meeting in the future (perhaps 2009), attracting professionals in oncology-related fields by offering CEUs/CMEs. Naturally, the meeting would be open to patient advocates, patients, caregivers, and interested others.

We help people with cancer make more informed treatment decisions.

P6-8: STRESS IN THE WAITING ROOM: WAITING FOR BREAST BIOPSY CAN BE MORE CHALLENGING THAN WAITING FOR INVASIVE TREATMENT

Elvira Lang and Nicole Flory

Beth Israel Deaconess Medical Center

Background: It is increasingly recognized that psychological support for patients diagnosed with tumors is important for coping and healing. It is also recognized that stressful medical experiences can adversely affect subsequent health behavior. The medical community often assumes that diagnostic procedures are not as challenging as actual tumor treatments even when these can be provided by minimally invasive surgical means. Even then interventional tumor treatments tend to take longer and are physically more invasive. Breast cancer diagnosis is a special circumstance because of the high overall incidence of malignancy and a higher likelihood that women know others who have been afflicted. This study took advantage of available pre-procedure surveys from women enrolled in prospective randomized trials in order to compare distress levels of women awaiting large core breast biopsy of undiagnosed lesions with those of women immediately prior to interventional treatment of known benign tumors (uterine fibroids) or known malignancies of the liver.

Methods: The study enrolled 253 participants referred to the radiology department of an urban, tertiary, university-affiliated medical center. Immediately prior to their procedures, women awaiting large core needle breast biopsy ($n=112$), uterine fibroid embolization ($n=71$), or hepatic chemo-embolization ($n=70$) completed 4 standardized psychological questionnaires: the State Trait Anxiety Inventories (STAI), Impact of Events Scales (IES), Center for Epidemiologic Depression Scales (CES-D), and Perceived Stress Scales (PSS). Data was analyzed via MANOVA and post-hoc Tukey HSD tests, and results were reported in means \pm standard deviations.

Results: Women prior to breast biopsy reported increased distress on all 4 measures compared to normative data for non-clinical samples. For the STAI, breast biopsy patients reported significantly higher anxiety (48 ± 12) than in the other groups ($p < 0.001$). Anxiety ratings were also higher prior to embolization of liver tumors (30 ± 16) than prior to embolization of benign fibroids (22 ± 14) ($p < 0.001$). On the IES, women awaiting breast biopsy (26 ± 16) and women undergoing chemo-embolization (27 ± 17) reported being significantly more affected by this life event than the fibroid group (20 ± 17) ($p < 0.05$). According to the CES-D, depressive symptoms were greater prior to breast biopsy (15 ± 12) and chemo-embolization (17 ± 11) than fibroid embolization (10 ± 10) ($p < 0.005$). Perceived stress ratings (PSS) were significantly higher prior to breast biopsy (18 ± 5) and chemo-embolization (scores 17 ± 7) than fibroid embolization (14 ± 8) ($p < 0.01$).

Conclusions: Anticipatory anxiety of women awaiting breast biopsy is considerable and exceeds that of women awaiting much more invasive treatments. Levels of depressed mood and distress of biopsy patients are comparable to those who are diagnosed with cancer. This study suggests that anticipatory anxiety around breast cancer testing, potential malignancy, and uncertainty of outcome can be more taxing than invasive treatments. Considering the adverse effects of preprocedural distress on sub-

sequent outcomes, the health care system is advised to promote adequate assistance. This has implications for training, staffing, and resource allocation of waiting rooms, particularly in mammography.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0153 and National Institutes of Health, National Center for Complementary and Alternative Medicine (1K24 AT 01074 and R01-AT-0002-07).

P6-9: ESTROGEN AND RESVERATROL AS REGULATORS OF THE RHO GTPASE RAC IN BREAST CANCER METASTASIS

Surangani Flanagan Dharmawardhane, Nicolas G. Azios, Linette Castillo-Pichardo, and Alina De La Mota-Peynado
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Metastatic breast cancer, where cancer cells spread by motile mechanisms and establish tumors at distant vital sites, is much harder to eradicate, and is often the cause of death from breast cancer. The female hormone estrogen has been shown to promote breast cancer while resveratrol, a structurally similar compound from grapes and red wine, is thought to act as a cancer preventive due to its antiestrogenic, antioxidant, antiproliferative, antigrowth, antiangiogenic, and anti-inflammatory effects. Data from our laboratory and others have shown that resveratrol at high concentrations may act as a breast cancer metastasis preventive by inhibiting breast cancer cell migration and invasion, extension of cell surface actin-rich structures that promote cell migration (lamellipodia), and activity of Rac. Paradoxically, our recent data demonstrate that resveratrol at low concentrations acts similar to estrogen and promotes cell functions relevant to breast cancer metastasis. Funded by BC010214 DoD/BCRP Idea award,

we previously demonstrated that Rac activity was a critical determinant of breast cancer metastatic efficiency (Baugher, et al., 2005. *Breast Cancer Res.* 7:R965-R974).

Therefore, we are testing the Hypothesis that estrogen and low concentrations of resveratrol promote breast cancer cell invasion and metastasis while high concentrations of resveratrol inhibit breast cancer cell invasion and metastasis via Rac-regulated mechanisms. Using estrogen receptor (ER) α (-) β (+) low metastatic MDA-MB-231 and ER α (-) β (-) high metastatic MDA-MB-435 human breast cancer cell lines, we show the following results. Estrogen and resveratrol at ≤ 5 μ M (effective dietary concentrations) increase cell proliferation, migration, invasion, lamellipodia extension, and Rac activity. However, resveratrol at concentrations >5 μ M inhibit cell proliferation, cell cycle progression, migration, invasion, lamellipodia extension, and Rac Activity. To determine a role for resveratrol in breast cancer progression in vivo, we tested the effect of dietary resveratrol in immunocompromised nude mice with mammary tumors established from fluorescent protein-tagged MDA-MB-435 breast cancer cells. Primary breast cancer progression and distant metastases were analyzed by quantification of the pixel intensity of fluorescently tagged breast cancer cells in situ using imaging modalities developed from the previously funded DoD/BCRP award (BC010214). Interestingly, dietary administration of 10 mg/kg resveratrol did not have a significant effect on primary mammary tumor growth compared to vehicle controls. However, resveratrol treatment at this concentration reduced distant metastases as determined by whole-body fluorescence image analysis. Studies are under way to determine the effective concentrations of resveratrol that inhibit or promote breast cancer metastasis. Therefore, this study is expected to generate important information on consumption of foods containing resveratrol for women diagnosed with breast cancer and survivors of breast cancer.

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HEALTH CARE DELIVERY

Poster Session P7

P7-1: PEER NAVIGATION IMPROVES ADHERENCE TO FOLLOW-UP DIAGNOSTICS AMONG KOREAN AMERICAN WOMEN WITH SUSPECTED BREAST ABNORMALITIES

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University of California, Los Angeles

Background and Objectives: Incomplete follow-up of potential breast abnormalities may contribute to disparities in breast cancer survival, especially in low-income, non-white populations. The purpose of this study is to test an intervention in the form of peer navigation to assist Korean American women who have been identified with a potential breast abnormality through the Breast Cancer Early Detection Program (BCEDP) and who have missed their first follow-up appointment (at-risk women). To recruit study participants, we have partnered with two community clinics in Koreatown that provide free breast cancer screening to low-income uninsured women through the Cancer Detection Program.

Methodology: We are testing our intervention in a randomized controlled trial, where half of the women are randomized to the control arm and half to the intervention arm. All subjects in the control arm are receiving usual care follow-up through the BCEDP case manager. They are recruited 6 months after their missed appointment to complete a telephone survey. Subjects in the intervention arm are recruited prospectively immediately after their missed appointment and receive peer navigation in addition to usual care. They complete the telephone survey at 6-month follow-up. Assessment includes adherence to follow-up procedures and related knowledge, attitudes, and quality of life. Chart reviews are being conducted to validate self-reported adherence to follow-up procedures. We are also collecting extensive process measures in the intervention arm including number and type of intervention activities requested and delivered to estimate the feasibility of institutionalizing the peer navigation program.

Results to Date: Since August 2005, we have identified 192 women who are eligible to participate in our study. Of them, 99 (51%) have been assigned to the control arm and 94 (49%) have been assigned to the intervention arm. Of those in the intervention arm, 76 (81%) have agreed to participate and have received various types of assistance from the peer navigator including: rescheduling of appointments (50%), reminder calls (81%), directions to the hospital (30%), translating (57%), filling out forms (55%), answering questions regarding the follow-up process (94%), and emotional support (72%). Among women who have completed the telephone survey 6 months after their missed appointment, 76% in the control group (44/58) and 95% in the intervention group (52/55) have reported completion of all recommended follow-up procedures ($p=.007$).

Conclusions: About 75% of women in the control group reported that they completed all follow-up procedures without assistance. However, completion rates were substantially higher in the intervention group. These preliminary results suggest that a peer navigator intervention is efficacious in this population. If chart reviews confirm our preliminary findings in the total sample, this intervention may assist in decreasing breast cancer disparities among Korean American women.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0676.

P7-2: PHYSICIAN CHARACTERISTICS ASSOCIATED WITH SERVING LIMITED-ENGLISH PROFICIENT BREAST CANCER PATIENTS

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Background and Objectives: The purpose of this analysis is to examine the characteristics of physicians providing breast cancer care to women in Los Angeles County, and to assess the personal, practice, and neighborhood characteristics of physicians who care for patients who are Limited English Proficient (LEP). This work is part of a larger study of the relative impact of individual and neighborhood-level ethnicity, language, and patient socioeconomic position on disparities in access to and quality of breast cancer care in Los Angeles County.

Methods: In this cross-sectional study, a self-administered survey was mailed to medical oncologists, radiation oncologists, and surgeons identified by a population-based sample of women with a new diagnosis of breast cancer. We queried about their personal and practice characteristics, including proportion of patients who are LEP (i.e., do not speak English well enough to give an adequate history). A total of 348 physicians completed the survey in 2004 (response rate: 77%). Using physician self-report data, we obtained information regarding physician personal characteristics (age, sex, race/ethnicity, specialty type, proficiency in a language other than English), and practice characteristics (setting type, size, availability of medical interpreters). Using 2000 Census data, we identified the percentage of population below the federal poverty level (FPL); percentage of the adult population who are high school graduates; percentage of the population who are Hispanic; and percentage who are foreign-born, and linked these to physician data by census tract associated with office loca-

tion. We tested for associations between physician report of their proportion of LEP patients and other physician (age, sex, race/ethnicity, specialty type, proficiency in a second language), practice (setting type, size, availability of trained medical interpreters), and neighborhood characteristics (proportions of Hispanic, foreign-born) using generalized linear modeling. Neighborhood-level poverty and education measures were excluded from the multivariate model due to high correlations with neighborhood ethnic composition.

Results: Physicians reported a mean of 17% of patients that were LEP (median: 10%), with Spanish the most prevalent language (88%). One quarter of physicians practiced in neighborhoods with majority Hispanic populations. In multivariate analysis, proportion of LEP patients was significantly associated with practicing in neighborhoods with higher proportions of Hispanic and foreign-born persons, physician self-report of speaking a language other than English, and County or Medical School practice setting, controlling for other physician characteristics (age, sex, race/ethnicity, specialty type), and practice characteristics (size, availability of trained medical interpreters).

Conclusion: Among physicians treating breast cancer patients in Los Angeles County, the proportion of LEP patients treated is higher among physicians who practice in neighborhoods with higher concentrations of Hispanic and foreign born persons, speak a second language, and practice in a County/Medical School setting. Cancer care research should consider structure of the healthcare setting, and neighborhood characteristics. Future work will assess the impact of LEP and use of interpreter services on measures of quality and outcomes of care.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0328; California Breast Cancer Research Program (7PB0126); University of California, Los Angeles; Center for Health Improvement in Minority Elderly/Resource Centers for Minority Aging Research; and National Institutes of Health, National Institute on Aging, National Center on Minority Health and Health Disparities (P30-AG-021684).

P7-3: ARE MANY COMMUNITY HOSPITALS UNDER-TREATING BREAST CANCER? LESSONS FROM 24,834 PATIENTS

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University of Miami, School of Medicine

Objective: To compare treatment patterns and long-term outcomes between teaching and community hospitals treating patients with infiltrating ductal carcinoma (IDC).

Methods: All IDC from the Florida Cancer Data System from 1994 to 2000 were examined.

Results: Overall, 24,834 operative cases of IDC were identified. Teaching hospitals treated 11.3% of patients with a larger proportion of stage III and IV disease (39.8% versus 33.0%). Five- and 10-year overall survival rates at teaching hospitals were 84% and 72%, compared to 81% and 69% at high-volume community hospitals and 77% and 63% at low-volume hospitals ($p<0.001$). In the MVA, this survival benefit was greater and independent of high-volume center status ($HR=0.903$, $p<0.001$).

Conclusions: IDC patients treated at teaching hospitals have significantly better survival than those treated at high-volume centers or community hospitals particularly in the setting of advanced disease. Poorer long-term outcomes for IDC at community hospitals appear due at least in part to decreased utilization of proven life-extending adjuvant therapies. These results should encourage community hospitals to institute changes in treatment approaches to invasive breast cancer to optimize patient outcomes.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0723 and James and Esther King Florida Biomedical Research Grant.

P7-4: BREAST CANCER CLINICAL TRIALS: GEOGRAPHICAL FACTORS IN ACCESS TO CARE

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University of California, San Francisco

Background and Objectives: Clinical trials are the major channel for translating treatment-related discoveries in breast cancer care into clinical practice. Enhanced participation by minorities in these trials is necessary to assess the effectiveness of advances in breast cancer care among major subpopulations and to ensure equity in the distribution of new treatment benefits. Despite inroads to increasing minority inclusion in breast cancer clinical trials, recent reports continue to demonstrate lower enrollment among African Americans, Asian Americans (AAs), and Latinos when compared to Whites. The primary objective of this abstract is to assess the accessibility of clinical trials to breast cancer patients and in particular, minority patients.

Methodology: We identified all breast cancer clinical trials conducted between July 2006 and June 2007 in four states with significant minority populations (California, Florida, Illinois, and New York) using the Physician Data Query (PDQ) from the National Cancer Institute (NCI). Included are treatment trials of oncologic drugs, biological agents, surgical and radiation procedures, and adjuvant and neo-adjuvant therapies. These trials were investigator-initiated or sponsored by cooperative groups or pharmaceutical companies.

Using geographic information software (ArcView 3.3), all sites were geocoded using the 2000 U.S. Census TIGER street file. We assessed the number of trials within each county, and the relationship to breast cancer cases by ethnicity. Preliminary analysis focused on California. Sites were mapped and yearly averages of breast cancer cases were estimated. Estimates were then used to generate a ratio of clinical trials for the total population and for each ethnic group. Correlation analysis was conducted to assess the relationship between breast cancer cases among each ethnic group and trial site at the county level.

Results to Date: We identified 344 clinical trials conducted in 260 sites. Almost half were classified as Phase 1 or 2; of these trials, the majority (40%) was Phase 2. Almost 80% of the trials were chemotherapy, hormonal, or a combination of both or tested a biologic therapy. The number of trials per site ranged from 1 to 29 trials.

Preliminary results for California indicated that 23 counties (out of a total of 58) had at least one trial site representing 85% of all breast cancer patients. The number of trials per county ranged from 1 to 72 with a mean of 17.34 (std=18.85). Overall there were 101 sites in California, with a mean of 3.98 trials per site (SD=3.46).

The ratio of sites to patients among counties with trials ranged from 1 site per 100 patients to 10 per 100 patients (in Alameda County). Los Angeles, the county with the largest number of breast cancer patients and the highest proportion of breast cancer cases among Latinos, had a ratio of 1 site per 100 patients. Further examination of the proportion of minorities indicates a lower ratio of trials to patients for Latinos. Correlation analysis indicated that as the proportion of Latino breast cancer cases increased, the ratio of sites decreased (coefficient -0.60, $p = 0.003$). This relationship was reversed among African Americans. Similar analysis will be conducted for Illinois, New York, and Florida.

Conclusions: Results will be used to describe the geographical distribution of trials and highlight counties in need of clinical trial expansion to achieve greater inclusion of all ethnic/racial groups.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0254.

P7-5: QUALITY BREAST HEALTHCARE FOR WOMEN WITH DISABILITIES

Marlene McCarthy and Kate McCarthy-Barnett
Rhode Island Breast Cancer Coalition

The Rhode Island Breast Cancer Coalition (RIBCC) conducted research to assess the delivery of quality breast healthcare for women with disabilities. Previous studies have found that the rates of breast cancer screening for women with disabilities are lower than for women without disabilities. When a woman has a disability, it does not make her any less of a woman. She is still at risk for breast cancer. Despite legal requirements, women with disabilities face barriers to quality breast healthcare and screening.

The objectives of this research involved exploring factors impacting screening experiences of women with disabilities including: documenting experiences accessing screening; identifying environmental factors impacting opportunities for screening; comparing screening experiences of women with and without disabilities; and assessing the delivery of quality breast healthcare.

The research was completed in two phases. In Phase 1, RIBCC conducted interviews with women with disabilities to assess experiences with breast cancer screening and a survey of all breast cancer screening providers in the state to determine factors impacting screening. In Phase 2, RIBCC conducted focus groups of healthcare providers to explore the delivery system of quality breast healthcare and surveyed women with and without disabilities to compare screening experiences.

The Phase 1 findings demonstrated that women with disabilities encounter physical, equipment, communication, technological, policy, and attitudinal barriers: Representative comments included "My arms were taped to the machine"; "I was never told when to hold my breath because no one knew sign language"; and "I had to change in the hallway with a sheet held up because the changing room could not fit a wheelchair." The Phase 2 findings demonstrated the need for improvement in the delivery of breast healthcare for women with disabilities and indicated significant differences among experiences between women with and without disabilities.

Based on the findings, RIBCC developed the *ACCESS for Women with Disabilities Program* to ensure women with disabilities are recognized as a vulnerable population at risk for breast cancer. This program is aimed at making changes in the delivery of quality breast healthcare for women with disabilities. The program components

include a new healthcare philosophy toward women with disabilities, accessibility policies, disability competencies to serve women with disabilities, assistive technology to enhance delivery, consumer advocacy, and community partnerships. RIBCC members with breast cancer provided leadership in design, implementation, and support program oversight.

The *ACCESS for Women with Disabilities Program* is successful in addressing breast healthcare for women who are traditionally underserved. Through the development of this program, RIBCC is making changes in system delivery for quality breast healthcare by ensuring equity for women with disabilities and providing the foundation to empower women with disabilities to become advocates for their healthcare rights. The components of the program influence standard of care, improve access to quality care, encourage systems change and accountability, and empower women with disabilities through information and training to make informed choices about their care. This initiative ensures that women with disabilities are recognized as a vulnerable population at risk for breast cancer.

P7-6: BREAST AND GYNECOLOGICAL HEALTH SCREENING EDUCATION AND TREATMENT PROGRAM FOR HOMELESS WOMEN

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¹SobreVivir - A Will To Live and ²St. Vincent's Catholic Medical Centers of New York

The Breast & Gynecologic Health Screening, Education and Treatment Program for Homeless Women, conducted at St. Vincent's Hospital Manhattan since 1998, is the foundation of the Elizabeth Monahan Homeless Women's Health Initiative. This initiative, which encompasses all aspects of health education for women, was named in memory of Elizabeth Monahan, former Program Director, who developed these vital services

The disenfranchised and fragile women served by this initiative have significant barriers to health care, including cancer diagnosis and treatment. Our SRO/Homeless Program works with these medically underserved individuals in shelters, drop-in centers, and SROs throughout New York City. The program identifies homeless women with breast, gynecologic, and other cancers and brings them into full diagnostic and therapeutic services through the Breast & Gynecologic Health Screening, Education and Treatment Program for Homeless Women

Health care is often ignored due to the enormous challenges that women in the target population encounter to survive: seeking the next meal and the next place to sleep, coping with chronic mental illness, achieving sobriety, struggling with drug addiction and domestic violence, and lacking job skills. The Breast & Gynecological Health Screening and treatment program component has succeeded in helping them concentrate on health screening, especially for breast and gynecological diseases, by providing mammograms, PAP smears, and gynecological examinations for patients, as well as diagnostic services and treatments, when indicated. With the available St. Vincent's on-site services and the Comprehensive Cancer Center of St. Vincent's, we have also been able to identify and treat women with other cancers as well. Our staff has developed clinical and communication skills needed to work with individuals who have suffered years of neglect.

The SRO/Homeless Program at St. Vincent's Hospital, which includes the Breast & Gynecologic Health Screening, Education and Treatment Program for Homeless Women, provides health care on-site at shelters, drop-in centers, and SROs throughout New York City. For all female clients at these sites, there is a special focus on women's health issues, particularly education on breast and gynecologic cancers. Our on-site presence ensures improved compliance and supports the long-term relationships that we develop with many of our clients. All women are taught the technique of breast self-exam (BSE). They also receive clinical breast exams and are offered gynecologic exams. If indicated, mammograms are arranged at St. Vincent's Comprehensive Cancer Center. For any abnormalities needing further follow-up, the necessary referral is made.

This work was supported by the Entertainment Industry Foundation.

P7-7: SPREADING "RAYS OF HOPE" IN WESTERN MASSACHUSETTS

Lucia Giuggio-Carvalho
To Life!

In 1993, at the age of 38, I was diagnosed with breast cancer while working as a nurse case manager on an oncology unit at Baystate Medical Center (BMC), a major hospital in western Massachusetts. I had a difficult time coordinating my care even though I was a nurse and understood the health care system. The question that emerged was how do women and their families with no medical background and understanding of the health care system manage to access and navigate their care? It was my personal struggle with the initial breast cancer diagnosis, treatment options decision, and identi-

fying support services that prompted this effort to make it easier for other women and their families with this journey.

Goal: Raise money for the development, support, education, outreach, and research locally in western Massachusetts through a fundraising walk for women diagnosed with breast cancer and their families.

Objectives and Mission of the Rays of Hope (ROH) Walk:

1. Support women with breast cancer and their families
2. Provide breast cancer education, outreach, and services in western Massachusetts
3. Support breast cancer research

Provide a “one-stop shopping approach” to information, services, outreach, and support in navigating the health care system

The hypothesis was founded on the concept that raising funds for specialized projects for women with breast cancer and their families will assist in raising awareness, education, and access to breast care services and also fund local research projects.

To begin planning for this walk, I developed a steering committee to organize the walk consisting of volunteers, family members, community leaders, medical professionals, breast cancer survivors, and representatives from the local media. I partnered with the nonprofit hospital where I worked to plan, manage, and distribute funds raised by ROH. A specialized fund was established with BMC.

The Medical Director for breast cancer services at BMC developed a Community Advisory Board with representation from hospital medical professionals, fundraising/development professionals, community leaders, breast cancer survivors, and community advocates including a member of the ROH steering committee.

Since 1994, ROH Walk has allocated funds to the Pioneer Valley Life Sciences Institute and the American Cancer Society for local research in the amount of \$1.1 million dollars. In total, ROH has raised over \$6.5 million dollars to support breast cancer services, education, community outreach, complimentary therapies, and research in western Massachusetts (1994–2007). Funds increased from \$45,000 and 6 projects in 1994 to \$833,000 and 32 different programs/projects in 2007. The majority of monies raised are from individual walkers/teams (under \$50,000/year from corporate sponsors). In 1994, there were 500 walkers and 25 volunteers. In 2007, 13,000 walkers and 800 volunteers participated.

In 2004, I advocated to establish an ROH Strategic Planning Committee on the 10th anniversary of the walk and became a member. The mission statement and goals were re-assessed and future strategies developed to meet the needs of the community.

The ROH volunteer steering committee along with our flagship sponsor organizes the walk each year with a volunteer community chair person—often a breast cancer survivor. In summary, the infrastructure is in place for continued breast cancer support and funding of local programs and research, and the interest and awareness from the community continues to grow.

This work was supported by Baystate Medical Center.

P7-8: ADVOCATES WORKING IN RESEARCH

Ann Hernick and Kathleen Ball

Breast Cancer Alliance of Greater Cincinnati

Breast cancer advocates who are trained, educated, and represent a patient constituency must be meaningfully involved in all aspects of decision making that affect breast cancer research. This is the only way to ensure that funds are effectively spent and adequately address the causes and prevention of breast cancer, develop optimal cures for breast cancer, and focus on the best possible means to eradicate the disease. At the University of Cincinnati's Breast Cancer and the Environment Research Center (BCERC), this model is indeed a reality. As background, in 2003, the National Institute for Environmental Health Sciences (NIEHS) and the National Cancer Institute (NCI) provided funding to develop 4 Breast Cancer and the Environment Research Centers. The 7-year grant specified that research scientists and breast cancer advocates work together to conduct human and animal/tissue culture studies and to translate findings to the public. These centers study the impact of prenatal to adult environmental exposures that may predispose a woman to breast cancer. The centers are: University of Cincinnati, Ohio; Fox Chase Cancer Center, Philadelphia, Pennsylvania; University of California, San Francisco, California; and Michigan State University, East Lansing, Michigan. The Cincinnati Breast Cancer and the Environment Research Center (Grant No: U01ES/CA012770) is a joint effort of researchers from the University of Cincinnati College of Medicine and Cincinnati Children's Hospital Medical Center and local breast cancer survivors and their advocacy organizations. We work together to better understand the development of the mammary gland and how/when it is affected by environmental agents using laboratory studies, learn more about how environmental and genetic factors affect the start of puberty in girls using population studies, and educate the community as well as public and policy makers about the research findings. Breast cancer advocates are involved in the research in the following ways:

Collaborate – With scientists to form active research partnerships both locally and in conjunction with the other BCERCs

Educate – Gain experience and knowledge about breast cancer research methods

Support – Create educational materials for study participants and their families

Communicate – Present research findings to the study families

Translate – Explain and interpret the research findings to the public

Publish – Articles about these activities and the research paradigm

Promote – National health messages with the BCERCs based on the research findings

Engage – In dialogue regarding the policy implications of the research findings

The perspectives of breast cancer advocates must be present everywhere that decisions regarding breast cancer research, prevention, and treatment are being formulated. A true partnership between the advocates and scientists is the most efficient and effective way to achieve the mutual goal of eradicating breast cancer because both parties bring distinct and valuable knowledge to the process.

This work was supported by the University of Cincinnati Breast Cancer and the Environment Research Center; National Institute of Environmental Health Sciences; and National Cancer Institute.

P7-9: GRASSROOTS ADVOCACY MAKES A DIFFERENCE!

Sandy Walsh and Michele Rakoff

California Breast Cancer Organizations

Since 1990 California Breast Cancer Organizations (CABCO) has worked with the California legislature in an effort to fulfill its mission: the eradication of breast cancer through education and advocacy. CABCO is a grassroots organization comprised of member organizations and individual members representing thousands who are affected by breast cancer. CABCO, working with other groups, has been a moving force in obtaining passage of significant legislation in the State of California and, as a board member organization of the National Breast Cancer Coalition, in Washington, DC.

California has been a leader in breast cancer screening programs, research, treatment, and cancer clinical trials as a result of legislation advocated for by CABCO, its members, and other groups in the state. The California Breast Cancer Act, passed in 1993, marked the first CABCO success in advocacy. This groundbreaking legislation levied a two cent tax on cigarettes:

- 50% for a breast cancer screening program for women over 40 (BCEDP)
- 45% for breast cancer research. An innovative CA Breast Cancer Research Program (CBCRP) was created to fund CA scientists and organizations doing research to alleviate the burden of breast cancer in the state
- 5% for the California Cancer Registry. The CA Cancer Registry initiated the CA Teachers Study, a prospective study following a cohort of 133,000 teachers.

To further fund breast cancer research, CABCO worked in 1994 to create a “check off” on state income tax forms that allows a tax-deductible donation to the CBCRP. In 2007 this provision was renewed for an additional 5 years.

With the establishment of these programs, there were provisions for advocates to play a role in their execution. Advocates serve on the governing bodies and on the advisory task forces for each of these programs.

As women were being diagnosed with breast cancer using the screening program with no provision for treatment, CABCO worked to obtain funding from the California Endowment to provide a treatment program for those diagnosed through the BCEDP and a federal program that had been created in the Centers for Disease Control and Prevention. In 2000, when this funding expired, CABCO advocates succeeded in obtaining state budgetary funding to continue the treatment program. At the same time CABCO members were working with NBCC to obtain passage of a federal treatment program. In 2001 CABCO worked with the California Governor to have the state budget include funding to “opt in” to the federal treatment program. This funding continues to be included in the state budget.

As another way to support research, CABCO initiated a clinical trials bill to promote cancer patient participation in clinical trials. This was successful in 2001. Insurers are now required to cover ordinary patient costs for those in clinical trials. A study by the University of California, Davis in 2004 validated that this legislation has had a positive effect on clinical trial participation by cancer patients.

Grassroots advocacy is effective! Dedicated, passionate CABCO advocates in California, working with the legislature and other organizations, have changed the face of breast cancer in California. Screening and treatment have been provided and creative research is being conducted. Trained, educated advocates with a passion to eradicate breast cancer make a difference.

DETECTION AND DIAGNOSIS I

Poster Session P8

P8-1: MULTIMEDIA NETWORKING AND WIRELESS TEST BED DEVELOPMENT FOR TELEDIAGNOSIS AND TELEMAMMOGRAPHY

Yu-Dong Yao

Stevens Institute of Technology

Providing mammographic services to women in underserved areas via telemedicine is very important. With remote computer-aided breast cancer detection and diagnosis, it has the advantage of higher penetration of women for cancer screening. In this research, we develop a new telemedicine scheme that is directed at the development, optimization, and evaluation of a new class of computer-assisted diagnostic system for telemedicine applications. In the telemedicine scheme, we use Internet and wireless transmissions to provide mammography to women in regions where physicians who specialize in diagnosing breast cancer are scarce. In this research, we examine the process of breast cancer diagnosis and the role of mammography, the use of medical image processing in mammography, the role of wireless communications in telemedicine services, and the performance impact of wireless systems on telemedicine.

A telemedicine network architecture was first developed. In this network architecture, there is a remote site and a central health office. A wireless transmission entity is in the center of the network. We have focused on the development of a software package to provide teleconferencing capability between the remote site and the central office and the development of a radio frequency (RF) transceiver with a multiband transmission capability. We have conducted the research through several project modules. (1) Development of an OpenH323-based software package to support multimedia teleconsultation (audio/video networking, graphic interference) in a telemedicine network. This project relates to the network architecture, multimedia networking, and socket programming. (2) Test bed control through handheld device and web interface. The project relates to wireless communications, wireless test bed, and software programming. (3) Mammogram database management and analysis. This project develops a database management system and handles mammogram processing. (4) Development of a tri-band RF front end for a wireless telemedicine test bed. This project investigates RF transmission requirements in telemedicine networks and conducts hardware and software development for the test bed. (5) Wireless and test bed performance evaluation through interactive web interface (Java). This project relates to wireless networking and java programming. (6) Security in wireless handheld device for medical applications. This project investigates security requirements in telemedicine and telemedicine applications and exams security protocols for telemedicine.

This research develops technologies to provide breast cancer detection services to women in remote areas and enhance health care delivery for cancer survivors.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0326.

P8-2: DUAL-SYSTEM TWO-VIEW APPROACH FOR COMPUTER-AIDED DETECTION OF BREAST MASSES ON MAMMOGRAMS

Jun Wei

University of Michigan

We are developing a computer-aided detection (CAD) system that combines a dual system scheme with a two-view fusion method to improve the performance for detection of both average and subtle masses on mammograms.

A total of 2336 mammograms from 745 patients including 800 normal mammograms from 200 normal patients were collected with IRB approval in this study. We randomly divided the cases with mass into two independent subsets to be used for training and testing in a cross-validation scheme. Normal cases were only used during the testing. Our system included two main processes: a dual system scheme and a two-view fusion scheme. The dual system scheme is consisted of two single CAD systems in parallel. The two single CAD systems had the same architecture, but one was optimized by using the masses on current mammograms (i.e., the mammogram exam that prompted the biopsy of the mass) and the other by the subtle masses on prior mammograms identified on retrospective review. An artificial neural network was trained to merge the mass likelihood scores from the two single CAD systems and differentiate true masses from false positives (FPs). After the mass candidates were detected by the dual system, a two-view fusion method that we developed was used to merge the information from different mammographic views. Mass candidates on the two-view mammograms were identified as potential pairs based on a regional registration technique. Morphological features, Hessian feature, correlation coefficient between the two paired objects, and spatial gray-level dependence texture features were used as input to train a similarity classifier that estimated a similarity score for

each pair. Stepwise linear discriminant analysis (LDA) with simplex optimization was used to select the most useful features and formulate the similarity classifier. Finally, another LDA classifier was used to fuse the score from the single-view dual CAD system and the two-view similarity score for each mass candidate. The detection performance of the CAD system was assessed by free response receiver operating characteristic (FROC) analysis.

When the regular CAD system trained on current mammograms was applied to the test sets, the average case-based sensitivities were 60% and 74% for masses on current mammograms and 29% and 45% for subtle masses on prior mammograms at 0.5 and 1 FPs/image, respectively. With the new dual-system two-view approach, the average case-based sensitivities were improved to 68% and 80% for current mammograms and 45% and 57% for prior mammograms at the same FP rates. The improvement in mass detection by the new system was statistically significant ($p < 0.05$) as estimated by JAFROC analysis.

Further work is under way to compare the observer performances with and without our CAD system.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0475.

P8-3: CORRELATIVE FEATURE ANALYSIS FOR MULTI-MODALITY BREAST CAD: IDENTIFYING THE CORRESPONDING LESIONS FROM DIFFERENT MAMMOGRAPHIC VIEWS

Yading Yuan, Maryellen Giger, Hui Li, and Charlene Sennett

University of Chicago

Background and Objectives: The purpose of the funded research project is to develop correlative feature analysis methods for integrating image information from multi-modality breast images, taking advantage of the information from different views and/or different modalities, and thus improving the sensitivity and specificity of breast cancer diagnosis. Identifying the corresponding image pair of a lesion is an essential step for this purpose. In this study, we present a correlative feature analysis (CFA) framework that differentiates the corresponding images from different views of a lesion from non-corresponding ones.

Brief Description of Methodologies: We developed a dual-stage method to segment mass lesions from the surrounding tissues. This algorithm utilizes an active contour model that maximizes an energy function based on the homogeneities inside and outside of the evolving contour. Prior to the application of the active contour model, a radial gradient index based method is employed to yield an initial contour close to the lesion boundary location in a computationally efficient manner. Instead of empirically determined criteria such as fixed iteration times, a dynamic stopping criterion is implemented to terminate the contour evolution when it reaches the lesion boundary.

Computer-extracted lesion features in this study were grouped into three categories: (1) margin and density features, (2) texture features, and (3) distance feature. For each pairwise set of features in different views, a Bayesian Artificial Neural Network (BANN) classifier was employed to merge each feature pair into a *correspondence metric*, which estimates the probability that the two images are of the same physical lesion, based on that specific feature. An effective subset of features were then automatically selected and merged with BANN to yield an estimation of probability of correspondence. Receiver operating characteristic (ROC) analysis was used to evaluate the performance of the proposed method.

Results to Date: We applied our method to a full-field digital mammographic database, from which 123 corresponding pairs and 82 non-corresponding pairs were constructed. Each pair consists of a cranio-caudal view and a medio-lateral view. Considering the most realistic scenario of lesion mismatch in clinical practice, the non-corresponding pairs were constructed from cases of the same patients but different physical lesions. In leave-one-out evaluation by lesion, the distance feature outperformed among all the individual feature, yielding an AUC (area under the ROC curve) of 0.81. The feature subset, which includes distance, gradient texture, and ROI-based correlation, yielded an AUC of 0.87. The improvement by using multiple features was statistically significant compared to single feature performance.

Conclusion: Our investigation indicates that the proposed method is a promising way to distinguish between corresponding and non-corresponding pairs. We are generalizing this method and applying it to differentiating corresponding and non-corresponding pairs from multi-modality breast images.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0726.

P8-4: INVESTIGATION OF SIMILARITY MEASURES FOR SELECTION OF SIMILAR IMAGES IN THE COMPUTER-AIDED DIAGNOSIS OF BREAST LESIONS ON MAMMOGRAMS

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Distinction between benign and malignant lesions can be difficult. To help radiologists in the diagnosis of breast cancers, researchers have been investigating computer-aided diagnosis, in which radiologists make a diagnosis by taking into consideration the output of computer analysis as "second opinions." Presentation of images with known pathologies that are similar to that of a new unknown case may be helpful to radiologists because radiologists' experience is often based on previous cases in their clinical practices and cases in the textbooks. The objective of this study was to develop a computerized scheme for selection of similar images that are useful to radiologists. The images of breast lesions used in this study were obtained from the Digital Database for Screening Mammography, which is a public database developed by the University of South Florida. We included 728 and 840 regions of interest (ROIs) with malignant and benign masses, respectively, and 457 and 644 ROIs with malignant and benign calcifications, respectively, in which the image qualities were adequate for this study. The contrast and the brightness of images were adjusted manually by a breast radiologist to facilitate viewing. For selection of images that are visually similar and useful to radiologists, subjective similarity ratings by breast radiologists were obtained to establish a "gold standard" for determination and evaluation of objective similarity measures. Three hundred pairs of masses and 300 pairs of clustered microcalcifications with various sizes and characteristics were selected, and 10 breast radiologists provided their subjective similarity ratings for each pair on a continuous rating scale between 0 and 1 corresponding to "not similar at all" and "almost identical," respectively. The objective similarity measures were determined based on four methods: pixel-value correlation of images, distance in the feature space, difference in the likelihood of malignancy levels, and output from an artificial neural network trained with the subjective similarity ratings and image features. The usefulness of the similarity measures were evaluated by the correlation coefficients between subjective similarity ratings and objective measures. The similarity measures based on the pixel-value correlation of masses were not very useful for this study. The correlations between the subjective ratings and the objective measure based on the distance in the feature space were moderate, 0.60 and 0.58 for the masses and microcalcifications, respectively. The correlations between the subjective ratings and objective measures based on the likelihood of malignancy were somewhat lower (0.53) for the masses and very low (0.21) for the microcalcifications. The similarity measures based on the outputs from the ANN provided relatively high correlations of 0.74 and 0.72 for both mass and calcifications, respectively. In our first observer study for evaluation of usefulness of the similar images in the distinction between benign and malignant masses, similar images were potentially beneficial to many cases, although the areas under the receiver operating characteristic curves without and with similar images were comparable. Further investigation is needed for evaluation of the usefulness of similar images.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0249.

P8-5: COMBINING MAMMOGRAPHY AND SONOGRAPHY FOR COMPUTER-AIDED DIAGNOSIS OF BREAST CANCER

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Background and Objectives: Computer-aided diagnosis models for breast cancer have traditionally used a single imaging modality, such as mammography. Now that it is becoming more common to use multiple imaging for breast cancer screening, computer-aided diagnosis models can take advantage of additional imaging information to improve diagnostic performance. The purpose of this study was to develop and evaluate computer-aided diagnosis models that include both mammographic and sonographic descriptors.

Methods: Institutional review board approval was obtained for this HIPAA-compliant study. A waiver of informed consent was obtained. Mammographic and sonographic examinations were performed in 737 patients (age range, 17–87 years), which yielded 803 breast mass lesions (296 malignant, 507 benign). Radiologist-interpreted features from mammograms and sonograms were used as input features for linear discriminant analysis (LDA) and artificial neural network (ANN) models to differentiate benign from malignant lesions. An LDA with all the features was compared with an LDA with only stepwise-selected features. Classification performances were quantified by using receiver operating characteristic (ROC) analysis and were evaluated in a train, validate, and retest scheme. On the retest set, both LDAs were compared with radiologist assessment score of malignancy.

Results: Both the LDA and ANN achieved high classification performance with cross validation (area under the ROC curve [Az] = 0.92 ± 0.01 and 0.90 ± 0.01 for LDA, $Az = 0.92 \pm 0.01$ and 0.90 ± 0.01 for ANN). Results of both

models generalized well to the retest set, with no significant performance differences between the validate and retest sets ($p > 0.1$). On the retest set, there were no significant performance differences between LDA with all features and LDA with only the stepwise-selected features ($p > 0.3$) and between either LDA and radiologist assessment score ($p > 0.2$).

Conclusions: Results showed that combining mammographic and sonographic descriptors in a CAD model can result in high classification and generalization performance. On the retest set, LDA performance matched radiologist classification performance.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0373.

P8-6: AUTOMATED MULTI-PARAMETER IMAGE ANALYSIS ALGORITHMS FOR QUANTITATIVE HISTOCYTOLOGY OF BREAST HISTOPATHOLOGY SAMPLES IMAGED BY MULTI-SPECTRAL MICROSCOPY

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Traditional methods for analysis of immunohistological staining are based on ad hoc regional properties like pixel intensities and morphological features (area, etc.). On the other hand, analysis based on cellular (cytometric) features offers significant advantages by quantifying antigens in biologically meaningful units (i.e., per cell). This mode of analysis quantifies antigens like flow cytometry but, importantly, retains tissue architectural and spatial information and the ability to differentiate antigens in different subcellular compartments. We have developed a system that allows histocytometric analysis of nuclear antigen immunostaining based on multispectral microscopy imaging, spectral unmixing, nuclei segmentation (delineation) based on a nuclear stain and subsequent association of nuclear antigen immunostaining with the delineated nuclei. To develop a system for analyzing antigens in cellular compartments, we have expanded on this work and present an approach to analyze antigen immunostaining in the cytoplasmic and plasma membrane subcellular compartments that use a "whole cell segmentation algorithm" (WCSA).

The WCSA involves multiple steps for robustly identifying nuclei from the hematoxylin channel, membrane markers from the cadherin channel, and cytoplasm from the cytokeratin channel. Nuclei from the hematoxylin channel are extracted by a three-step process. In the first step, we delineate potential cell clusters by applying a novel fast graph-partitioning level set algorithm. This is followed by applying a simplified version of Parvin et al., iterative radial-voting algorithm to identify seeds in the cellular clusters. These seeds are subsequently used in a label propagation (or watershed) algorithm to extract individual cells from the clusters. A multi-channel version of the graph-partitioning algorithm is jointly affected on all three channels to correctly identify locations of cytokeratin staining (representing cytoplasm). This results in accurate boundaries from the Ck+ regions as cues from other channels (e.g., nuclei from the hematoxylin channel) are used to "fill up" disconnected or highly concave regions near Ck+ boundaries that arise from a lack of Ck+ staining. This is followed by variational thresholding, connected component analysis and binary thinning to identify locations of potential membrane markers from the cadherin channel (representing plasma membrane). Disconnected membrane regions are "joined" by any suitable edge-linkage algorithm. Application of WCSA to images of immunostained tumor specimens allows cytometric quantification of antigens, such as HER2 and p-AKT that are clinically relevant in breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0325.

P8-7: RECENT ADVANCES IN MICROWAVE DETECTION OF BREAST CANCER: A COMPACT LOW-COST REFLECTOMETER AND ENHANCED SENSING MODALITY THAT COUPLES DIELECTRIC AND ELASTIC PROPERTIES CONTRASTS

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University of Wisconsin, Madison

The primary scientific aim of this project is to develop sensitive, compact, and low-cost reflectometers that can be combined with antenna arrays to achieve a field-deployable approach to microwave breast imaging, especially for scenarios where the size, weight, and cost of established screening or diagnostic techniques is prohibitive. Reflectometers are circuits that measure the ratio of reflected waves to incident waves. Tissue-penetrating radar systems rely upon reflectometers to acquire tissue-dependent microwave scattering signatures. Array-based signal processing techniques can be applied to the measured signals to generate a microwave image of the breast.

We have designed and constructed a compact and low-cost wideband reflectometer for use in an array-based microwave breast imaging system. Mixer-based cascaded bridge-T attenuators serve as compact planar wave separators in our design instead of directional couplers. We have also developed associated calibration procedures for the reflectometer-antenna system that improve measurement sensitivity and repeatability. The performance of our reflectometer prototype in detecting backscattered signals from simple laboratory phantoms compares favorably with more expensive and bulky commercial vector network analyzers. These accomplishments represent a critical step toward making promising microwave imaging technology available to the clinician, particularly in underserved populations or in the field.

In addition, we have explored the feasibility of a hybrid sensing modality that exploits contrasts in both the dielectric and elastic properties of malignant and normal breast tissue. Conventional microwave imaging modalities seek to exploit differences in the dielectric properties of malignant and normal breast tissues while elastic-properties contrast—namely the increased stiffness of cancerous tissues relative to normal tissue—serves as the physical basis for elastography. In our proposed hybrid modality, low-frequency acoustic or mechanical excitation is applied to induce tissue-dependent displacements in the heterogeneous breast interior while low power microwave signals are transmitted into breast, and the scattered microwave signals are measured using an antenna array. The induced tissue deformations modulate the scattered microwave signals detected by the receiver. Thus, the received microwave signals carry information related to both the dielectric and elastic properties of breast tissues thereby potentially increasing the diagnostic value of the microwave image.

We have conducted computational studies of microwave scattering in mechanically excited breast tissue using efficient multi-physics (mechanical and electromagnetic) simulation methods and numerical breast phantom testbeds. The tissue-dependent dielectric properties values assumed in our breast phantoms are taken from a recent large-scale dielectric spectroscopy study of breast tissue. In our simulations, the observed contrast in the harmonic backscatter of malignant and normal fibroglandular masses in the breast was larger than the contrast in the fundamental backscattered signals associated with unperturbed tissue. These results demonstrate the potential for enhancing the overall sensitivity of microwave breast cancer detection through the introduction of a Doppler component in the microwave scattered signal that depends on elastic properties.

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P8-8: MICROWAVE IMAGING FOR BREAST CANCER DETECTION: TIME-DOMAIN INVERSE SCATTERING WITH BASIS FUNCTIONS

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Recent research has shown that the dielectric properties of breast tissue at microwave frequencies are sensitive to certain physiological factors of clinical interest, such as water content, temperature, and vascularization. Thus, microwave imaging can be used to exploit indicators of malignancy associated with these factors of interest. Microwave modalities promise to complement established diagnostic tools through the use of low-power non-ionizing radiation, advanced imaging algorithms, and low-cost hardware. Active microwave imaging techniques such as microwave tomography (MT) and tissue-penetrating radar (TPR) are noninvasive and avoid the need for breast compression. Preliminary results show that these techniques have the potential to detect early stage breast cancer.

Active microwave imaging involves transmitting microwave signals into the breast using an antenna array and measuring the scattered microwave signals. A variety of strategies can be employed to create an image of an underlying microwave frequency characteristic of the breast. For example, in MT, inverse scattering algorithms are applied to the scattered microwave signals for the purpose of estimating the spatial distribution of dielectric properties within the breast. However, MT is a computationally intensive approach that requires solving a nonlinear and ill-posed mathematical problem. Consequently, it may take on the order of days to generate a single 3-D image. In TPR, signal processing algorithms are applied to the scattered microwave signals to detect, localize, and characterize scattering targets within the breast based on their radar cross section. While TPR does not solve a complicated inverse problem like MT, its accuracy is limited by the ability to model the effects of microwave propagation through the breast tissue, which are expected to change from patient to patient.

The research conducted under the support of this training grant has addressed critical issues that will enable practical clinical implementation of these two techniques. We

have developed two algorithms for calibrating TPR imaging techniques. The first estimates the location of the breast surface from scattered signals recorded across the antenna array while the second estimates patient-specific propagation effects within the breast. The breast surface location data also improve the performance of MT. We have addressed the issue of computational complexity in MT by expressing the distribution of dielectric properties within the breast with a linear combination of patient-specific basis functions. These patient-specific basis functions reduce the dimension of the inverse problem and allow a greater number of measurements to be considered for a given amount of computational storage. Combined with the use of hardware acceleration technology, this accomplishment has reduced the computation time for MT from days to hours. Similar to conventional microwave tomography, our patient-specific basis function approach produces relatively low-resolution images due to the spatial blurring associated with the ill-conditioned inverse problem. We have proposed a new MT approach where we assume that the high-resolution details can be represented with a small number of fine-scale basis functions. Sparse approximation methods are used to solve for the coefficients of these basis functions, and we show through computational models that increased imaging accuracy can be achieved relative to conventional MT.

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P8-9: IN VIVO DETECTION AND CHARACTERIZATION OF MAMMARY TUMORS IN A MURINE MODEL SYSTEM

Eric Blue,¹ Eric L. Bradley,¹ Nicholas Kenney,² Jianguo Qian,¹ Joannathan Sutton,¹ Amir Yazdi,¹ Robert Welsh,¹ Andrew Weisenberger,³ Stan Majewski,³ Stephen Schworer,¹ and Margaret Somosi Saha¹
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Effective diagnosis and treatment of breast cancer rely on a precise and detailed understanding of the cellular-molecular alterations that give rise to an oncogenic phenotype. Recent advances in cellular and molecular biology have resulted in significant progress in identifying many of the molecules implicated in the genesis and progression of mammary cancer. Substantial progress has also occurred in the use of new imaging technologies that allow investigators to follow mammary tumor development in vivo and noninvasively with high resolution and sensitivity. Continued progress requires fusing molecular biology and imaging with the goal of imaging the metabolism of specific tumor-related molecules. Toward that end, we have developed a small animal imaging system consisting of a position-sensitive gamma camera that can detect low levels of ¹²⁵I-labeled ligands in real time with significant sensitivity and resolution. We have applied the detector system to understanding the progression of mammary tumors by imaging the dynamic uptake of Na-¹²⁵I through the sodium iodide symporter (NIS). Using the mouse mammary tumor virus (MMTV) model, a population of 40 MMTV mice exhibiting 59 tumors was analyzed. These experiments have demonstrated not only the sensitivity and the efficacy of the detector but also the following: (1) gamma camera images of Na-¹²⁵I uptake correlate precisely with NIS protein based on whole-mount immunohistochemistry; (2) Na-¹²⁵I is potentially a very promising radiotracer for detecting nonpalpable tumors at the earliest stages of development (as small as 3 mm); (3) tumors display profound heterogeneity both temporally and spatially in uptake of iodine; (4) tumors fall into three groups based upon distribution of signal—center-to-edge, multi-spot, and ring—and pattern correlates with tumor size and age with smaller tumors falling into the first category; (5) tumors show no relationship between the speed of tumor development and the initial size or pattern of the tumor; (6) patterns of uptake (and loss) are dynamic, suggesting different characteristics of cells within the tumor, with regions of greatest change being those with first evidence of uptake; and (7) other nontumor tissues, in particular other mammary glands, show differences in pattern of uptake when a tumor is present. Taken together, these results suggest that radiiodide imaging is a promising in vivo method for monitoring the changes associated with tumor development such as changes of tumor size, pattern, and aspects of gene-specific metabolism over both short and long durations. Our results also indicate that the classifiable heterogeneity present in dynamic gamma camera images may correlate with specific patterns or signatures of gene expression that, in turn, indicate tumor subtype and progression. These data may allow investigators to develop an effective and sensitive system of in vivo imaging of molecules and metabolism that reflect the molecular signature of a tumor in real time. Our system may provide a means for early detection, ideally even a precancerous state before malignancy develops, and a method to assess the overall state of a tumor with the goal of predicting the best therapeutic regime and following the efficacy of the therapy in real time by examining specific molecular targets.

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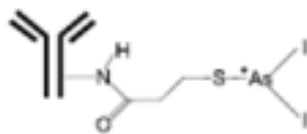
P8-10: BREAST TUMOR DETECTION AND TREATMENT USING BAVITUXIMAB LABELED WITH ARSENIC RADIONUCLIDES

Ralph P. Mason,¹ Marc Jennewein,¹ Xiankai Sun,¹ Guiyang Hao,¹ Marcus Jahn,² Matthew A. Lewis,¹ Dawen Zhao,¹ Linda Watkins,¹ Sean O'Kelly,³ Padmakar V. Kulkarni,¹ Alex Hermanne Alex Hermanne,⁴ Frank Rösch,² and Philip E. Thorpe¹

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Background and Objectives: This project aims to generate a novel approach for detection and therapy of advanced disseminated breast cancer based on fundamentally novel concepts. The first critical component exploits the discovery of a novel naked antibody, 3G4, which targets phosphatidylserine (PS) expressed on tumor vasculature. In collaboration with Peregrine Pharmaceuticals, this agent has been chimerized and is currently in clinical trials as bavituximab. Normally, PS exclusively resides on the cytosolic leaflet of the plasma membrane. However, in tumors PS becomes externalized on the vascular endothelium and upon some tumor cells. Bavituximab not only targets tumors, but also induces vascular damage and tumor regression with minimal accompanying toxicity. The second key component is the application of diverse arsenic radionuclides suitable for imaging based on positron emission tomography (PET; ⁷²As T_{1/2} 26 h, ⁷⁴As- T_{1/2} 17.8 days) and radio immunotherapy (RIT; ⁷⁷As (T_{1/2} 38.8 h, E β 226 keV)). New isolation methods provide arsenic radionuclides in useful yields and labeling antibodies derivatized with N-succinimidyl-S-acetylthioacetate (SATA) generates viable products of high specific radiochemical and biological activity. Aims include i) optimizing bavituximab labeling with arsenic radionuclides for imaging, biodistribution, and radio immunotherapy; ii) detecting diverse primary breast tumors and evaluation of metastatic spread using radio arsenic labeled bavituximab.

Results: Initial studies show a labeling time of 30 min generated [⁷⁴As]bavituximab with a labeling yield >99.9%. Radio-HPLC confirmed the absence of any aggregates or free ⁷⁴As. Incubation of [⁷⁴As]bavituximab in undiluted fetal bovine serum up to 72 hr confirmed in vitro stability of [⁷⁴As]bavituximab with no aggregation or breakdown products. Immunoreactivity comparing ELISA of [⁷⁷As]bavituximab, unlabeled unmodified bavituximab and [⁷⁷As]rituximab as a negative control demonstrated that SATA modification and radioarsenic labeling caused no reduction of immunoreactivity.



Conclusions: Since phosphatidylserine expression appears to be a universal characteristic of stressed vasculature in tumors, we hypothesize that studies in rodents will be directly translatable to the clinic. The radionuclides of arsenic were selected because their long half-lives are consistent with the long biological half lives of antibodies in vivo and because their chemistry permits stable attachment to antibodies. We believe that radio arsenic-labeled antibodies will be useful for the imaging, detection, and therapy of cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0475 and National Cancer Institute (U24 CA126608).

P8-11: DUAL BIOLUMINESCENCE TOMOGRAPHY-SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY FOR STUDIES OF METASTATIC BREAST CANCER IN PRE-CLINICAL MODELS

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Background and Objectives: This project aims to develop a novel imaging system to facilitate studies of breast cancer metastasis in small animal models. The bioluminescence tomography modality is optimized for quantitative assessment of primary tumor burden and detection of distant, small metastases (less than 10³ cells). With suitable radiopharmaceuticals, the SPECT modality is well-suited for studies of tumor vasculature and microenvironment, both of which are recognized as important factors in metastatic potential.

Results: Two in-house cameras have been developed for this project. The TC253 camera, developed using FPGA methodology developed by us, was completed in 2006 and a report was accepted for publication in fall 2007 (Soesbe et al. *IEEE Trans Nuc Sci.* 54(5), 1516-1524, 2007). The TC253 camera was discovered to have an undocumented noise floor due to spurious charge generation that was subsequently multiplied in the serial amplification registers. A cooled e2v camera was also developed for testing and comparison.

Rather than upgrading to the next generation TI TC 247 sensor, the decision was taken to purchase 4 commercial Andor Luca cameras with this chip. This funding was independent of the project but has advanced it considerably.

Since multiple USB cameras were not supported by the manufacturer's software, a system was needed for using all cameras simultaneously. Software and methods were

developed for converting the Advanced Radiological Sciences Rocks 4.2 computation cluster to a Windows XP based cluster where each node controls an emCCD camera. The computational nodes are connected in a star topology with a frontend through a Gigabit switch, and the system can be rapidly reconfigured from computational cluster to data acquisition cluster. Images are published across the high-speed network to the frontend for image reconstruction, processing, and analysis. This high-speed network is private and not accessible from the campus network.

To facilitate image reconstruction for the SPECT component, a robot was temporarily coupled to the imaging system. Using a point source of radioactivity, the transfer matrix for the complete imaging volume was measured. This data is incorporated in a variety of image reconstruction methods.

While the initial design was to use the same CCD camera for both modalities, our research has concluded that each imaging component is optimized with a different camera. Although the systems are independent, integration is accomplished by mounting the optical and gamma ray cameras on the same gantry. In this configuration, co-registration is not needed, and data from the optical imaging component can be used to constrain the nuclear medicine image.

Conclusions: We have surmounted considerable technical difficulties to achieve our goal of dual-modality imaging, associated with the high performance requested of our combined device. We have explored the advantages and limitations of available, current technology and demonstrated ways to achieve both nuclear medicine and light-emission imaging in a compact device at the cost of producing two modalities; we have also developed software to reconstruct the images. We have demonstrated detection and visualization of metastases of <1,000 cells using bioluminescence and are currently initiating dual-modality imaging using nuclear medicine techniques combined with light emission.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0551 and National Cancer Institute (U24 CA126608).

P8-12: AUTOMATION AND PRECLINICAL EVALUATION OF A DEDICATED EMISSION MAMMOTOMOGRAPHY SYSTEM FOR FULLY 3-D MOLECULAR BREAST IMAGING

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Our lab has developed a novel single photon emission computed tomography (SPECT) system for uncompressed dedicated molecular breast imaging or "mammothography." Using a compact, high performance gamma camera, the versatile positioning gantry allows for fully three-dimensional (3D) imaging anywhere within a hemispherical volume about the pendant breast and overcomes physical proximity restrictions of standard clinical gamma cameras or compact systems without 3D motion (Figure 1). The overall objective of this proposal is to fully automate and optimize the performance of the system for enhanced semi-automated clinical imaging. First, a retrospective study of >100 clinical MRI scans of uncompressed breasts was conducted to compile a database of pendant breast measurements and surface renderings. Analysis of the wide variety of breast volumes and shapes observed reinforced the need to automate the SPECT acquisition orbits for auto-contouring. Ribbon laser ranging sensors and associated hardware for positioning automation sensing were acquired and successfully bench tested. Software and hardware system implementations are currently in progress. A human observer based contrast-detail study was performed in an effort to evaluate the limits of object detectability for the SPECT system under various imaging conditions. A novel, geometric contrast-resolution phantom was developed that can be used for both positive (hot) and negative contrasts (cold). Results show little statistically significant difference (p<0.05) between simple versus complex 3D acquisition trajectories or whether the signals appeared hot or cold, indicating that data acquisition with the system is quite robust. We anticipate that fully automated molecular breast imaging will improve detection and potentially in vivo characterization of early-stage breast cancer.

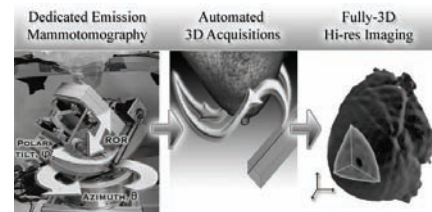


Figure 1. Dedicated Emission Mammotomography

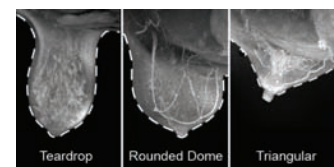


Figure 2: Sample Breast MRI images and shape classifications

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0765 and National Institutes of Health (R01 CA096821).

P8-13: MOLECULAR STAGING OF BREAST CANCER USING PET

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Background and Objectives: Standard screening misses up to 40% of breast cancers. Breast cancer cells express a novel breast cancer-associated glycoprotein, mammaglobin A (MamA), which is expressed in 80%–100% of breast cancers and up to 90% of lymph node metastases, but not in normal tissues. Peptide nucleic acids (PNAs) are hybridized strongly and specifically to RNA and DNA, do not induce RNase H and so do not destroy their bound target, and are unlikely to interact with cellular proteins that normally bind negatively charged molecules. It is our hypothesis that a MamA-PNA analogue can be created that will allow in situ and invasive breast cancer detection.

Methodologies: To test our hypothesis, we aim to (1) synthesize a ^{64}Cu -anti-MamA-PNA IGF-IR analogue, along with appropriate control sequences, at very high specific activities suitable for imaging breast cancer MamA mRNA overexpression in vivo; (2) in an orthotopic mouse model of human breast cancer, determine if the antisense analogue for imaging MamA mRNA expression is effective in imaging the cancer with high sensitivity and specificity, using high-resolution PET/MRI imaging; and (3) evaluate a fluorescent anti-MamA-PNA analogue for its ability to detect MamA mRNA in biopsy specimens of in situ and invasive breast cancer versus benign breast tissue.

To achieve our aims, we are preparing correct sequence and control peptide-antisense PNA chimeras labeled with ^{64}Cu radionuclide for noninvasive PET imaging of MamA gene expression in breast cancer. We will obtain tissue distributions in an orthotopic mouse model of human breast carcinoma and in human benign and malignant breast tissue to evaluate the sensitivity and specificity of the agents for MamA mRNA. We will also perform high-resolution PET/MRI imaging in tumor bearing mice to evaluate the radiolabeled peptide-PNA chimeras for noninvasive detection of breast cancer.

Results to Date: The correct antisense sequence has been synthesized. We are in the process of synthesizing the control sequences. We have analyzed total RNA from MDA-MB-468 and ZR-75-1 estrogen receptor-positive cells for MamA expression. By RT-PCR we were able to detect mammaglobin A in both cell lines with higher expression in ZR-75-1 ($\text{Ct}=17.4$) than in MDA-MB-468 ($\text{Ct}=32$) cells.

Conclusions: It is our hope that the MamA-antisense-PNA probe that we are preparing will detect human breast cancer with high sensitivity and specificity. These pre-clinical and human ex vivo studies will position us optimally to investigate the clinical usefulness of this probe to aid in the early detection of human breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0559.

P8-14: POTENTIAL OF FLUORESCENCE SPECTROSCOPY AS AN ADJUNCT DIAGNOSTIC TOOL TO IMAGE GUIDED CORE NEEDLE BREAST BIOPSY

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University of Wisconsin, Madison

Current core needle biopsy for breast cancer diagnosis has limited sampling accuracy. Incorporating an optical sensor into the biopsy needle to identify tissue types at the needle tip prior to biopsy could potentially improve its sampling accuracy. The goal of this study has been to develop a prototype optical sensor based on ultraviolet-visible (UV-VIS) fluorescence spectroscopy and to demonstrate its feasibility for breast cancer detection during core needle biopsy.

A side-firing fiber optic probe that works compatibly with a 9-gauge vacuum-assisted biopsy needle has been developed for in vivo fluorescence spectroscopy during the core needle breast biopsy. A total of 73 patients undergoing image-guided percutaneous biopsy have been enrolled in this clinical study, and a total of 121 biopsy samples with accompanying histological diagnosis were analyzed to determine the potential of fluorescence spectroscopic characterization of breast tissue pathology.

The measured tissue spectra were analyzed using Partial Least Squares analysis and represented using a set of principal components (PCs) with dramatically reduced data dimension. These PCs accounted for a large portion of data variances and characterized the difference in spectral features observed between malignant and nonmalignant breast tissue samples, including that characteristic of tryptophan, collagen, reduced nicotinamide adenine dinucleotide (NADH), and retinol. For nonmalignant tissue samples, a set of PCs that account for the largest amount of variance in the spectra displayed statistically significant correlation with the percent tissue composition, suggesting increased collagen and NADH fluorescence and decreased tryptophan and retinol fluorescence in fibrous tissues relative to adipose tissues. For all tissue samples, a set of PCs were identified using a Wilcoxon rank-sum test as showing statistically significant differences between (a) malignant and fibrous/benign, (b) malignant

and adipose, and (c) malignant and nonmalignant breast samples. These PCs were used to distinguish malignant from other nonmalignant tissue types using both linear and nonlinear Support Vector Machine (SVM) classifier. For the sample set investigated in this study, the linear SVM provided a cross-validated sensitivity and specificity of 70.4% and 86.8%, respectively, for discriminating between malignant and fibrous/benign samples and 81.5% and 81.0%, respectively, for discriminating between malignant and adipose samples. The nonlinear SVM classifier with a radial basis function kernel provided a cross-validated sensitivity and specificity of 81.5% and 86.8%, respectively, for the discrimination between malignant and fibrous/benign samples and 74.1% and 81.0%, respectively, for discriminating malignant from adipose tissue samples.

The study demonstrates the feasibility of performing fluorescence spectroscopy during clinical core needle breast biopsy, and the potential of this technique for identifying breast malignancy in vivo. This study also set the precedent for the next-generation probe design and a larger scale clinical study. It is expected that with the accrual of clinical data, the value and capability of the fluorescence spectroscopy technique as an adjunct real-time diagnostic tool to assist the biopsy of malignant breast lesion can be fully tested.

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P8-15: NEUTRON-STIMULATED EMISSION COMPUTED TOMOGRAPHY FOR DETECTION OF BREAST CANCER

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Breast cancer is the leading type of cancer to affect women in the United States. Screening mammography, which is the only FDA-approved tool for breast cancer screening, has several limitations in its anatomic approach in identifying abnormalities. First, it requires the lesion or calcification to be in an advanced stage of development. Second, detected lesions are classified as benign or malignant based on morphological features such as shape and size, making it difficult to categorize lesions that are not already visible. Third, screening mammography requires good contrast, which is often difficult to achieve in women with dense breasts.

We propose to overcome these limitations through a new spectroscopic screening technique called Neutron Stimulated Emission Computed Tomography (NSECT) that is sensitive to metabolic changes seen very early in tumor development. Several studies have shown that malignant tumors exhibit changes in trace element concentrations much before masses or calcifications begin to appear. Detecting these changes could potentially enable diagnosis of breast cancer at very early stages.

NSECT uses a spectroscopic approach to quantify element concentrations in breast tissue at a molecular level. Neutrons striking an atomic nucleus stimulate it to emit gamma radiation that is unique to the emitting element. Measuring the energy and quantity of the emitted gamma rays allows direct determining of the element concentration in tissue. Ratios of element concentrations detected are then used to classify the tissue as benign or malignant. Preliminary data suggest that this technique has great potential in developing into an effective screening tool for early diagnosis of breast cancer.

This project aims at evaluating the efficacy of NSECT as a noninvasive technique for breast cancer diagnosis. Efficacy is evaluated by determining the accuracy of detecting trace elements in the breast and measuring the corresponding dose delivered. Patient dose is of significant concern when using neutrons because at energies of interest to NSECT, neutrons are known to damage the body 10 times more than x-rays. However, preliminary experiments have shown that it may be possible to achieve clinical doses as low as 10 mSv. A primary objective of this project is to reduce dose further to allow NSECT to be more easily accepted as a screening tool.

Detection accuracy and dose reduction are being evaluated through Monte-Carlo simulations in GEANT4. The NSECT acquisition system has been simulated in the GEANT4 environment to allow system evaluation and development without requiring a physical neutron beam. Two types of gamma detectors have been simulated and evaluated in GEANT4, high-resolution germanium detectors and lower resolution bismuth germinate detectors. Anatomic phantoms for benign and malignant breast tissue tumors have been designed and implemented in GEANT4. Spectral data have been obtained from these simulated phantoms and compared with data from benign and malignant breast biopsy specimens for validation of the simulations. Results of spectroscopic simulations indicate that NSECT has the ability to detect clinically relevant concentrations of trace elements in breast tissue for diagnosis of breast cancer. The simulation is currently being expanded to facilitate tomographic scanning for determining the optimal scanning strategy and developing the MLEM reconstruction algorithm for two-dimensional tomographic imaging of breast tumors.

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P8-16: IN VIVO SMALL ANIMAL NEAR INFRA-RED FLUORESCENCE IMAGING WITH PRIOR ANATOMICAL INFORMATION

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Applications of Near Infrared (NIR) Fluorescence imaging for the molecular and biochemical imaging has been a subject of recent interest. The assessment of angiogenesis and vasculature in breast cancer is of importance as a key indicator of survival and response to therapy. The fluorescence imaging of these angiogenic factors have the potential to provide valuable information about the molecular changes on cellular and/or tissular levels.

One of our objectives was to quantitatively compare the reconstructed fluorescent images obtained from semi-infinite reflection geometry and cylindrical transmission geometry with and without the assistance of ultrasound. The second objective was to quantitatively compare the reconstructed fluorescent images from different boundary conditions.

We have built a frequency-domain system to study the fluorescence properties of different biomolecules in breast tumors. In this study, a laser diode at 690 nm was used as the excitation light and sequentially multiplexed delivering light into the tissue via 9 source fibers. 14 detector fibers were selectively arranged in semi-infinite reflection geometry circumscribed by our own-designed hand-held probe in order to collect fluorescence light from the tissue. Furthermore, image reconstruction was attained by developing a chi-square fitting technique to reconstruct the structural parameters (lesion size, location, etc), and a dual-zone mesh technique that reconstruct the functional parameters (fluorophore concentration). The fluorescence data was normalized with the scattered data for the final image reconstruction. Knowing the structural and anatomical information in prior significantly improved the fluorescence image reconstruction. A commercial ultrasound transducer was rarely used as adjunctive tool for anatomical information.

After building the system's hardware and developing our own imaging algorithms, we were able to reconstruct images from the fluorescence data, and studied the effects of different data collection geometries, boundary conditions and probe configurations for optimizing the information from the target. We successfully imaged few nanomoles (nM) of Cyanines (Cy5.5 and Cy7), depths in the range of 2-3 cm into the turbid medium. We had an opportunity to image a unique angiogenesis marker; Vascular Endothelial Growth Factor (VEGF) conjugated with Cy7 (fluorescence peak at 776nm), developed by our cancer biologist and collaborator Dr. Joseph Backer. The conjugate facilitates the visualization of angiogenesis development and adjacent tumor growing sites. Recently, we were able to image in vivo small tumors in mice at 1 cm deep; subjects were injected with the VEGF/Cy7 conjugate. Our image reconstruction result showed a quantified Cy7 fluorophore concentrations ranging from 40-50nM, which in agreement with the injected dose to the mice. All the small animal imaging results were corroborated with phantom studies for validation of our technique.

We have been able to image small-size tumors at their early stages with concentrations of fluorophores at few nano-mole levels. This is a substantial step that clearly represents a pivotal point for the development of new techniques suitable for the discovery of breast cancer at its earliest stages. The ultimate goal is to image early angiogenesis changes of the malignant process and therefore early detection and treatment of breast cancers.

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P8-17: CHARACTERIZATION OF BREAST TUMORS WITH NIR METHODS USING OPTICAL INDICES

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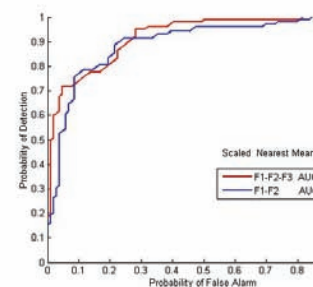
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In this work, we evaluated the characterization efficiency of optical properties of breast tumors using in vivo data obtained by near-infrared (NIR) spectroscopy. Our evaluation criterion is based on statistical classification techniques. Three features, namely, relative blood concentration, oxygen saturation, and the size of the tumor were used to characterize benign and malignant tumors.

A continuous wave NIR spectrometer is used to collect the optical data. The spectrometer includes a probe. In the center of the probe there is a 3-wavelength light emitting diode (LED). The probe consisted of one multiwavelength LED as a light source and 8 silicon diodes as detectors. The detectors surround the LED with a 4 cm radius. The light intensity from the detectors was adjusted to be approximately 1 volt and calibrated with a phantom with known absorption and scattering coefficients.

This study includes two centers, namely, the Abramson Family Cancer Research Institute, Department of Radiology of the Hospital of University of Pennsylvania (HUP), and the Department of Gynecology of Leipzig University (DGLU). HUP provided 24 patients with malignant and 64 patients with benign tumors. DGLU provided 20 patients with malignant and 6 benign tumors.

The performance of the proposed set of features (relative blood concentration, oxygen saturation, and the size of the tumor) was evaluated using various classifiers on 44 patients with malignant tumors, and 70 patients with benign tumors. Figure 1 presents the receiver operating characteristics (ROC) curves for all three features, and the best two features, namely, relative blood concentration and oxygen saturation using a scaled nearest mean classifier. In Figure 1, F1 presents the relative blood concentration, F2 presents the oxygen saturation, and F3 presents the patient age. The observed area under the ROC curve for F1-F2-F3, and F1-F2 are 0.9098, and 0.9001, respectively with a significance level of 0.05. Our results suggest that the relative blood concentration, and oxygen saturation has potential to differentiate malignant and benign breast tumors with a relatively high accuracy. This set of features can potentially be incorporated into diagnostic systems to aid physicians for breast cancer diagnosis.



ROC curves for F1-F2-F3 and F1-F2 using scaled nearest mean classifier

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P8-18: A GENERALIZED LEAST-SQUARES MINIMIZATION METHOD FOR NEAR INFRARED DIFFUSE OPTICAL TOMOGRAPHY

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Diffuse optical tomography (DOT) has the potential to become a noninvasive and nonionizing diagnostic imaging technique for breast cancer imaging. DOT uses near-infrared light to illuminate the breast, generally through use of fiber optic bundles, and the diffusely transmitted light is collected from the tissue surface. To get functional images of the breast, one needs to use advanced model-based reconstruction methods with these measurements. Accurate modeling of the scatter-dominated transport process leads to a nonlinear transport model, which is difficult to solve. A thorough understanding of existing methods and the development of improved novel computational approaches are vital in the process of making diffuse optical imaging a viable clinical tool.

In this work, an improved optical image reconstruction method is developed, which takes into account the possible errors associated with the collection of data. This formalized framework uses the generalized least-squares (GLS) approach where the aim is to match the experimental data with the modeled data using a least-squares minimization with inclusion of a regularization term to stabilize this ill-posed problem. Most least-squares approaches become special cases of this GLS approach. As a part of this work, a computationally efficient approach to dramatically reduce the size of the matrix to be inverted in cases where the number of imaging parameters are much larger than the boundary data is developed. This algorithm reformulates the inversion approach, within most least-squares approaches, to allow the inversion to be based upon the size of the data rather than the number of imaging field parameters, and this is useful for most three-dimensional (3D) imaging situations. This algorithm improved the computational speed by at least a factor of three.

Prior spatial and spectral information about the tissue is used to improve the spatial resolution and quantification of physiological parameters of tissue. It is illustrated that incorporation of spatial prior information reduced the image error by at least a factor of two. It is shown that the GLS approach was able to give reasonable estimates (with in 20% error limit) of spectrally derived tissue functional properties even in cases of highly noisy experimental phantom data. Traditional reconstruction algorithms are not able to converge to any meaningful solutions in these cases.

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P8-19: MRI-COUPLED FLUORESCENCE MOLECULAR TOMOGRAPHY FOR BREAST CANCER IMAGING

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The primary objective of this research is to develop and test a novel technology to image internal breast physiology to aid in detection, treatment planning, and treatment monitoring of breast cancer patients. Current imaging modalities used for breast cancer detection and characterization suffer from high false positive rates and in some cases low sensitivity. Furthermore, no standard exists for monitoring treatment efficacy during a chemotherapy regimen. Patients often complete the lengthy regimen before learning the treatment was ineffective and a new treatment track must be followed. A noninvasive method to identify failed treatment regimens earlier in the process may result in a shift to a more effective treatment course. The fluorescence imaging system developed under this grant may aid in tumor characterization and treatment monitoring for breast cancer.

Fluorescence Molecular Tomography (FMT) is an emerging technology to image fluorescent drug activity in biological tissue. Light waves between the red and infrared wavelengths penetrate over 10 cm through biological tissue. By illuminating a breast with laser light after the patient has been injected with a fluorescence dye and measuring the fluorescent light that escapes the surface of the breast with sensitive instruments, it is possible to build an image of the drug distribution or fluorescence activity in the breast. A particularly exciting aspect of this technique is that targeted drugs may be used to mark different physiological processes, such as over-expression of protein receptors on malignant cells. Images thus reveal where in the breast the targeted process is most active and can help differentiate benign and malignant tumors.

FMT produces low resolution images compared to standard clinical modalities such as x-ray computed tomography (CT) and magnetic resonance imaging (MRI) due to the highly scattered photon fields and relatively sparse measurement sampling of the tissue volume. Imaging sensitivity drops for deeper tumors making it more difficult to image through larger tissue volumes. However, these challenges may be overcome by coupling the FMT system into conventional imaging systems and incorporating the highly resolved conventional images into the fluorescence activity image formation process.

This grant contributed to the construction and development of a state-of-the-art FMT imaging system that couples into the bore of conventional MRI systems for simultaneous fluorescence and MR image acquisition of the human breast. Mathematical algorithms responsible for transforming the collected data into meaningful images of fluorescence activity have also been developed and tested. These algorithms incorporate the highly resolved MR images as templates to guide the formation of the fluorescence activity images. Using relatively simple breast-sized tissue simulating phantoms, it has been demonstrated that imaging fluorescence activity of Indocyanine green, a nonspecific fluorophore approved for human use, is possible at relatively low tumor:background drug uptake contrasts (about 1.5 to 1), even for deeply embedded tumors. Simulation studies in more complex breast domains with heterogeneous optical properties show similar results and confirm that FMT without the aid of MRI is nearly intractable, though can be quite accurate as long as the system is coupled with the MRI data.

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P8-20: SUBHARMONIC ULTRASOUND CONTRAST IMAGING OF BREAST MASSES: PRELIMINARY RESULTS

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To improve on the characterization of breast masses, we prospectively compared the accuracy of grayscale subharmonic imaging (SHI) to grayscale ultrasound (US), power Doppler imaging (with and without contrast) and mammography for the diagnosis of breast cancer using histopathology or follow-up as the reference standard.

The study was approved by the Thomas Jefferson University's Institutional Review Board and all subjects gave written informed consent prior to enrollment in the study. Fourteen women with 16 biopsy-proven breast lesions participated in this pilot study. In SHI pulses are transmitted at one frequency but only echoes at half that frequency (i.e., the subharmonic) are received. A Logiq 9 scanner (GE Healthcare, Milwaukee, Wisconsin) was modified to perform grayscale SHI (transmitting/receiving at 4.4/2.2 MHz). Pre-contrast scans (grayscale US and power Doppler) were followed by contrast-enhanced power Doppler imaging and grayscale SHI. A blinded reader assessed diagnosis on a 6-point scale. Sensitivity, specificity, accuracy, and ROC curves were computed for mammography, grayscale US and power Doppler (pre and post contrast) as well as SHI. Finally, digital clips were acquired of each injection and SHI time intensity curves determined within each lesion using Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland). SHI perfusion estimates were deter-

mined using a linear relationship previously established in a canine model [Forsberg et al., *J Ultrasound in Medicine*, 2006].

Of the 16 lesions, 4 (25 %) were malignant. Mammography had a sensitivity of 100% and a specificity of 20%. Pre-contrast imaging achieved a sensitivity of 50% and a specificity of 92%, while contrast-enhanced power Doppler produced 75% and 75%, respectively. SHI had a sensitivity of 75% and specificity of 83%. All US modes produced higher specificities than mammography ($p < 0.04$). There were no significant differences in specificity among US modes or for sensitivities ($p \geq 0.50$). The area under the ROC curve for the diagnosis of breast cancer was 0.64 for baseline, 0.67 with contrast-enhanced power Doppler, 0.76 with mammography, and 0.78 with SHI ($p > 0.20$). SHI perfusion estimates were significantly lower in malignant than benign lesions (1.93 ± 0.03 versus 1.70 ± 0.09 mL/min/g; $p = 0.04$). Contrast enhancement was better with SHI than with power Doppler (100% judged "good" or "excellent" versus 44 %; $p = 0.004$).

In conclusion, a contrast-specific US imaging technique, SHI, has been investigated for in vivo breast imaging. SHI appears to improve the diagnosis of breast cancer relative to conventional US and mammography (albeit based on a very limited number of subjects studied) and as such SHI could provide a major improvement to breast imaging and patient care.

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P8-21: EXCITATION ENHANCED IMAGING FOR BREAST CANCER DETECTION: IN VITRO AND IN VIVO RESULTS

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To improve on the detection of breast cancer, a novel ultrasound (US) contrast imaging technique called Excitation Enhanced Imaging (EEI) has been developed. EEI employs two acoustic fields: a low-frequency, high-intensity US field (the excitation field) to actively condition (or increase the size of) contrast microbubbles and a second higher frequency, lower intensity regular imaging field applied shortly afterward to detect the enhanced contrast scattering [Forsberg et al., *Ultrasonic Imaging*, 2005]. We have investigated the efficacy of EEI in vitro and in vivo.

Five different US contrast agents were tested in vitro: Sonazoid (GE Healthcare, Oslo, Norway), Definity (BMS Medical Imaging, N Billerica, Massachusetts), Therimage (Focus Therapeutics, Media, Pennsylvania), QFX (Nanfang Hospital, Guangzhou, China), and Optison (GE Healthcare, Princeton, New Jersey). An excitation frequency of 1.1 MHz at an amplitude of 1.2 MPa was used with a 16-cycle pulse length at PRF of 2 Hz. Imaging frequencies of 2.5–10 MHz were investigated for concentrations of 0.02–80 μ L/L at ambient (22°C) and physiological (37°C) temperatures to determine the change in scattered signal strength before and after the excitation pulse (i.e., the enhancement obtained with EEI) at fundamental and harmonic frequencies. A new zero-thickness interface model was used to simulate the dual-pulse imaging mode associated with EEI and compared to the in vitro measurements. A Logiq 9 scanner (GE Healthcare, Milwaukee, Wisconsin) with a 3.5C curved linear array and an AN2300 digital US engine (Analogic Corp., Peabody, Massachusetts) with a P4-2 phased array transducer (Philips Medical Systems, Bothell, Washington) were modified to perform EEI on a vector-by-vector basis in fundamental and pulse inversion harmonic grayscale modes. In vivo EEI was tested in 4 rabbits and 4 dogs.

At a 2.5 MHz imaging frequency, Sonazoid produced 10 dB of enhancement at 22°C, which reduced to 5 dB at 37°C. Conversely, Optison created 1 dB of enhancement at 22°C, which increased to 9 dB at 37°C. This enhancement reduced to 3 dB when the concentration was increased from 0.05 to 0.5 μ L/L. While no enhancement was found for Definity at any of the concentrations studied, QFX produced approximately 17 and 14 dB of enhancement at the fundamental and harmonic frequencies (5 and 10 MHz), respectively. Initial simulation results indicate that the shell elasticity plays a vital role in the growth as well as dissolution of the bubbles. While results at an imaging frequency of 7.5 MHz were somewhat in agreement with measurements, the enhancement was unrealistically high (20–35 dB). Further work is ongoing to improve upon the model. Somewhat disappointingly, only 1–4 dB of enhancement was produced by EEI in vivo.

In conclusion, up to 17 dB of enhancement can be achieved with EEI in vitro. This imaging mode could potentially have a large impact on breast cancer management by increasing the sensitivity of US imaging for the presence of cancer. Moreover, patients with benign lesions, who might otherwise be subjected to unnecessary biopsies due to limitations in the currently used diagnostic techniques, may be identified using EEI. However, EEI appears to be quite sensitive to changes in temperature and microbubble concentration, which may explain the reduced enhancement observed in vivo. Further research is needed to clarify this issue.

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P8-22: ULTRASOUND IMAGING OF BREAST CANCER

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Although generally useful in cancer detection, diagnosis, and monitoring of treatment, there remains a need to improve the accuracy of ultrasound (USO) when deployed within the setting of breast cancer. One way to improve USO imaging is to administer a chemical contrast agent that can enhance the magnitude of the USO signal, particularly when this can be done in the immediate vicinity of the cancerous tissue. One means of directing chemical agents to cancerous tissue is to incorporate molecular features that are specifically recognized by cancer cells compared to healthy cells. Overexpression of integrin adhesion molecules on breast cancer cells destined to undergo metastasis provides an opportunity to target them by utilizing the RGD peptide motif as an address component for a given cargo.

This presentation first describes our chemical synthesis of the RGD address molecule linked to (loaded with) a cargo that is anticipated to be able to enhance USO imaging signals. Until the latter is demonstrated to be effective in this regard and can thereby be submitted for patent protection, its specific composition must remain proprietary. Thus, at this point our specialized cargo will simply be abbreviated as "CA" for contrast agent. The second part of the presentation describes our attempts to establish an in vitro assay to assess USO imaging of human cancer versus normal tissues with and without the benefit of chemical contrast agents. Given the success of USO imaging in the in vivo setting, the difficulties that we are encountering while attempting to develop in vitro methods even at the level of our control studies have been somewhat surprising.

Since we had a significant delay in the onset of our program after obtaining funding, we have recently requested a 1-year, no-cost extension so as to be able to continue with our investigations in this area. If need be, we are prepared to examine this concept within the context of our RGD-CA probe by deploying an in vivo model implanted with human breast cancer cells.

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P8-23: NONINVASIVE MONITORING OF BREAST CANCER DURING NEOADJUVANT CHEMOTHERAPY USING OPTICAL TOMOGRAPHY WITH ULTRASOUND LOCALIZATION

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We investigated the feasibility of using optical tomography in the near-infrared spectrum (NIR) combined with ultrasound (US) localization (NIR/US) to monitor tumor vascular changes and pathologic response during chemotherapy for locally advanced breast cancer. Eleven female patients were studied during treatments with a combined imager consisting of a commercially available US system coupled to a NIR imager. Tumor vascular content was assessed based on total hemoglobin (tHb) concentration and volume obtained from NIR data. Percentage blood volume index (PBVI) was calculated as the product of tHb concentration and volume using the pre-treatment tHb image as the baseline. At treatment completion, pathological assessment revealed three response groups: complete or near-complete responders A, partial responders B, and non-responders C. The mean PBVI of groups of A, B, and C at the treatment completion were 29.1% ($\pm 6.9\%$), 46.3% ($\pm 3.7\%$), and 86.8% ($\pm 30.1\%$), respectively (differences statistically significant, $p < 0.04$). At the end of cycle two, the PBVI of group A is noticeably lower than the partial ($p = 0.091$) and non-responder groups ($p = 0.075$) with moderate statistical significance. Our findings indicate that NIR/US using PBVI can be used during chemotherapy to monitor tumor vascular changes. It also may distinguish pathologic response during treatment allowing for tailoring therapies to response; this requires more patients to confirm.

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P8-24: CONTRAST ENHANCEMENT FOR THERMAL ACOUSTIC BREAST CANCER IMAGING VIA RESONANT STIMULATION

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University of Florida

Our objective is to develop enhanced contrast thermal acoustic imaging (TAI) technology for the detection of breast cancer by combining amplitude-modulated (AM) electromagnetic (EM) field excitation, resonant acoustic scattering, and advanced signal processing techniques. EM-induced TAI combines the merits of both EM stimulation and ultrasound imaging while overcoming their respective limitations. EM imaging provides excellent contrast between cancerous and normal breast tissue,

but the long wavelengths provide poor spatial resolution. Conventional ultrasound imaging possesses very fine millimeter-range spatial resolution but poor soft tissue contrast. While EM-induced TAI possesses great promise, the thermal acoustic signals tend to be weak. However, when the tumor is excited into resonance via EM stimulation, the effective acoustic scattering cross-section may increase significantly based on predictions for microsphere-based ultrasound contrast agents. Such an increase would make the EM-induced TAI technology a very promising candidate for routine breast screening.

To induce the resonant response from the tumor, we consider various approaches including, for example, AM continuous wave (CW) EM stimulation, where the modulation frequency range contains the predicted resonant frequencies for a distribution of tumor sizes and contrast ratios. The carrier frequency of the EM stimulation can be fixed and chosen for the best penetration and heat absorption. Moreover, adaptive image formation algorithms are devised to achieve high resolution and excellent interference and noise suppression capability.

Preliminary experiments serve to verify the feasibility of sensing the acoustic pressure from a tissue phantom excited at resonance. We have finished the design of a tissue phantom that mimics the electrical and mechanical properties of a tumor for use in the excitation system. The initial experiment has been designed to verify acoustic resonance of the tumor phantom by electromagnetic excitation. Currently the properties of the tumor phantom (dielectric constant, conductivity, and mechanical resonant frequency) are being evaluated as a function of temperature and time (age of phantom).

We have devised adaptive and robust methods of reconstruction (ARMOR) for thermoacoustic tomography. Examples based on a numerically simulated 2-D breast model and two sets of experimentally measured data from human mastectomy specimens demonstrate the excellent performance of ARMOR: high resolution, low-side lobe level, and much improved interference suppression capability.

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P8-25: EFFECT AND CORRECTION OF CHEST-WALL LAYER ON OPTICAL TOMOGRAPHY FOR BREAST CANCER IMAGING WITH PRIOR ULTRASOUND ANATOMIC INFORMATION

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In our clinical studies on the detection of breast cancer by near-infrared (NIR) diffusive optical tomography, we have found that more than 30% of the breast lesions are located closer to chest wall, which consists of mixtures of muscles and bones. When the chest wall is shallow, the obtained optical measurements are often distorted. As a result, the fitted optical parameters may deviate from true value and reconstructed image may be inaccurate and distorted. In this paper, we applied the Monte Carlo (MC) method to analyze the effect of chest wall on the optical measurements and developed the reconstruction algorithm that can correct the distortion of chest wall with a prior ultrasound anatomic information.

The breast tissue and chest wall are modeled as two layers in the simulation. A new photon tracking method using MC simulation is developed. The detected photon can be separated into several groups according to the propagation path of the photon. When there is a target inside the medium, the photons can be separated into four groups according to the combination of whether the photon enters the target or second layer. The percentage of the weight for each group, which indicates the influence of this group to the total measurements, can be calculated by summing the weights of all detected photons for each group and then dividing the total weight.

Our simulation shows that the chest-wall layer has much larger contribution on the measurements than the target when the breast-tissue layer thickness is less than 1.5 to 2.0 cm deep depending on bulk optical properties. Different references, such as optical property-matched and depth-unmatched reference, depth-matched and property-unmatched reference, and property-unmatched and depth-unmatched reference, can cause errors on reconstructed image. The property-matched and depth-matched reference provides the best result with error less than 5%, and the depth-matched and property-unmatched reference provides the worst results with error larger than 60%.

In the second step, we combine the solutions of diffusion equations for two-layer structure with the linear perturbation for image reconstruction. The diffusion equations were solved using the Fourier transform approach for two-layer medium. In our new image reconstruction method, the weight matrix now modified by calculating Green function and scattered field using the solution obtained from the two-layer model. Thus the weight matrix now takes into account the presence of the second layer. The layer thickness can be read from U.S. image, and the optical properties of both the tissue layers can be fitted from the measurements. To evaluate our reconstruction method, we have performed a series of phantom experiments. The reconstructed images with layered model are always 10-20% better than the reconstructed images reconstructed with semi-infinite model in the absolute value, and the shape of image with layered model is consistently better than the image using the semi-infinite model. The clinical experiment is under way.

In conclusion, the chest wall has great influence on the NIR optical measurements when the breast tissue thickness is less than 2.0 cm. It is necessary to have a matched reference for correct image reconstruction. The new tomography method based on two-layer model analytical solution can improve the light quantification accuracy and image shape.

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P8-26: ACOUSTIC INVERSE SCATTERING FOR TASK-SPECIFIC BREAST SONOGRAPHY: DEVELOPMENT OF NON-IONIZING METHODS FOR MICROCALCIFICATION DETECTION IN HIGH-RISK POPULATIONS

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Background: Microcalcification detection is the hallmark of mammography as a breast cancer screening modality. For technical reasons, ultrasonic detection of all mammographically-visible microcalcifications has been problematic. In clinical ultrasound, high frequencies must be used to resolve microcalcifications below 200 micrometers. Unfortunately, ultrasonics above 10 MHz suffer from appreciable attenuation in soft tissues, and depth of penetration is limited. Transmission diffraction tomography, while well-suited for the geometry of the breast, is inherently insensitive to scattering caused by small, hard inhomogeneities. A more general form of acoustic inverse scattering is therefore needed for microcalcification detection and localization by ultrasound.

Objective: An advanced scalar inverse scattering theory developed by Colton, Kirsch, and others in the inverse scattering community can determine the shape of scatterers with size on the order of the wavelength. In addition to size and number, the morphology of breast microcalcifications is an important diagnostic indicator. Our hypothesis is that the linear sampling method (LS), when augmented with a method

for estimating the inhomogeneous Green's function for wave propagation in the breast, can be translated to an acoustic imaging system to detect, localize, and characterize microcalcifications in breast phantoms using data from the far-field scattering measurements.

Study Design: A prototype system for imaging microcalcifications (20 to 400 micrometers) will be developed using available hardware technologies and software development of algorithms derived and implemented from the LS method for acoustic inverse scattering. The region-dependent Green's function for background tissue inhomogeneities will be estimated from images acquired using traditional diffraction tomography. Inverse scattering algorithms will be tested using simulations, and the system will be characterized by imaging morphological phantoms of the breast with small inclusions of appropriate dimension and material properties to represent microcalcifications. Resolution limits, contrast sensitivity, and image degradation due to attenuation will be evaluated for illumination within the 5 to 15 MHz range. Performance will be determined using task-based measures for ideal observers.

Progress: In the initial 4 months of this study, we have assembled the appropriate data acquisition system to perform these experiments. Algorithm development and integration have commenced.

Impact: Ultrasound imaging of the breast is accepted at present only as an adjunct to mammography in the diagnosis of symptomatic breast disease. Ultrasound is not currently used for screening of breasts for non-palpable lesions; but with proven sensitivity and specificity, this non-ionizing ultrasonic method has the potential to allow earlier and more frequent breast screenings in at-risk patients. If breast compression can be avoided by the imaging technique, then ultrasound also represents a less stressful procedure for the patient. Breast ultrasound may be the only available technique for screening in the radiologically dense breasts of women under 40. Advances in early detection have already increased survivability, and there is reason to believe that improvements in breast screening with microcalcification-sensitive ultrasonic imaging will continue this trend.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0640.

MAGNETIC RESONANCE IMAGING I

Poster Session P9

P9-1: DETECTION AND EVALUATION OF EARLY BREAST CANCER VIA MAGNETIC RESONANCE IMAGING: STUDIES OF MOUSE MODELS AND CLINICAL IMPLEMENTATION

Sanaz Arkani Jansen, Suzanne Conzen, Thomas Krausz, Gillian Neewstead, and Gregory Karczmar
University of Chicago

Objectives: The early detection of breast cancer is a major prognostic factor in the management of the disease. In particular, detecting breast cancer in its pre-invasive form as ductal carcinoma in situ (DCIS) improves prognosis greatly compared with invasive tumors. However, because the natural history of DCIS is not well understood there is a clinical concern that DCIS may be overdiagnosed and overtreated. The goals of this project are to: (1) characterize the MR kinetic and morphologic findings of DCIS in women and compare with benign lesions and other malignant cancers, (2) develop techniques to detect early mammary cancer in mice, and (3) study the development and progression of early mammary cancer in mice by performing longitudinal MRI studies of development of DCIS and transition to invasive cancer.

Methods:

Clinical Studies: The contrast media uptake and washout curves were mathematically analyzed. We analyzed the kinetic characteristics of 79 pure DCIS lesions by nuclear grade and mammographic presentation and also compared the kinetic characteristics of DCIS with other malignant and benign lesions.

Murine Studies: Twelve SV40 Tag transgenic mice were selected for imaging to determine whether MRI of early cancer, including DCIS, was feasible. MR images were compared with histopathology. To study the progression of DCIS, eight mice were selected for serial imaging every 2 weeks from ages 12–18 weeks.

Results to Date:

Clinical Studies: The kinetic characteristics of pure DCIS lesions were associated with mammographic presentation but not nuclear grade. Invasive cancers exhibited significantly larger contrast uptake and stronger washout compared with DCIS lesions, which in turn showed considerable overlap with benign lesions.

Murine Studies: MRI was able to detect 17/18 small (~1mm) tumors and 13/16 ducts distended with DCIS greater than 300 microns in diameter (Figure 1). DCIS lesions developed at an average age of 14.5 weeks and small tumors at 17.3 weeks. Four of 8 mice did not progress from DCIS to invasive cancer within the study period.

Conclusions: To our knowledge, this is the first demonstration that MRI can detect early murine mammary cancers, including DCIS, in vivo. We found that some DCIS lesions did not progress significantly during the study window. With the results presented here, MRI could be used to assess efficacy of therapies on cancers at all stages of disease (in situ, early, and advanced) rather than only the advanced, palpable tumors that are typically used in current murine therapy trials.

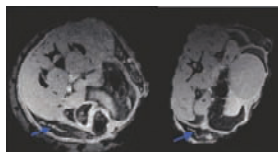


Figure 1. MRI of two mice showing ducts distended with DCIS 300 microns in diameter (arrows)

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0329 and Segal Foundation.

P9-2: NON-INVASIVE PHOSPHORUS-31 MAGNETIC RESONANCE SPECTRAL CHARACTERIZATION OF BREAST TISSUE ANOMALIES USING PATTERN RECOGNITION AND ARTIFICIAL INTELLIGENCE

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¹University of Arkansas at Little Rock, ²Amrita Institute of Medical Sciences (Cochin, India), ³National Center for Toxicological Research, ⁴Vanderbilt University Medical Center, and ⁵University of Arkansas for Medical Sciences

Background and Objectives: It is highly desirable to develop a noninvasive technique that could detect and reliably interpret images from suspicious regions within a woman's breast. Due to the pervasive nature of breast cancer in society, this is a very timely proposal that could have a significant impact on women's health. It is known that there is a high degree of correlation between the chance of complete recovery and early detection. Phosphorus-31 (P-31) magnetic resonance imaging (MRI) signals produce signals similar to nuclear magnetic resonance (NMR) spectrum used in chemistry to identify molecules.

Methodologies: The MR signal is obtained from the naturally occurring Phosphorus-31 element within a small volume of tissue within a woman's breast. All data were acquired on a 1.5 T General Electric Signa Horizon system using a home-built solenoid phosphorus coil. The phosphorus coil consisted of 1/4 inch wide copper tape wrapped on an acrylic tube 13.6 cm in diameter. Pre- and postcontrast clinical RoDEO (ROtating Delivery of Excitation Off-resonance) images were acquired to localize the lesion. A slice (1 cm³ voxel) containing the lesion was chosen and used

for the spectroscopy exam. This exam used a phase-encoded pulse acquire technique. Each spectral data set required about 30 minutes.

Results: We were able to show that P-31 MR signals contain some diagnostically useful information, and coupled with our neural network procedures, could be used to noninvasively diagnose malignant from nonmalignant tissue. Although we had a very limited number of patients [six] (see Figure 1), we were able to "find" two patients with malignant tissue. One patient was confirmed with a biopsy, but the second patient was suggested by analysis of the data.

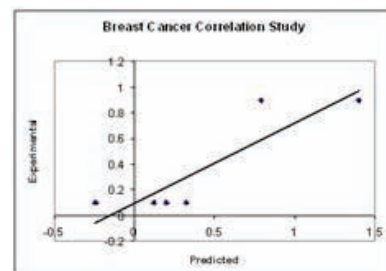


Figure 1: Correlation plot of the experimental results vs. predicted results from the artificial neural network for 6 patients. The arrow indicates possible malignant tissue in patient 6, not verified with biopsy.

Conclusions and Impact: Although we were not able to study a larger sample of patients, we were able to show that this procedure has the potential to noninvasively diagnose malignant tissue within the breast. With further studies, it should be capable of differentiating the different types of malignancies present. This would impact the prescribed urgency of treatment and survivability.

Future Research: We have made a new contact at [UAMS] (Dr. Larry Suva). He will obtain new samples of breast tissue for analysis. We will look at P-31 and the Gd isotope used in RoDEO studies. It should lead to improved prediction of the noninvasive malignant and nonmalignant tumors in breast tissue.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0491.

P9-3: MRI STUDY OF UNINVOLVED BREAST TISSUE FOR PATIENTS WITH LOCALLY ADVANCED BREAST CANCER UNDERGOING PRE-OPERATIVE CHEMOTHERAPY

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University of California, San Francisco

The purpose of this retrospective study was to better understand the relationship between breast cancer tumors and their normal "host" tissue, by studying the MRI characteristics of normal tissue in patients with invasive cancers. Since there is a strong association between the tumor morphologic patterns on MRI with both treatment outcome and survival, we hypothesized that the imaging phenotype reflects tumor growth patterns that are influenced by the normal host tissue.

Methods: A total of 68 women were previously enrolled in a breast MRI study at our institution and underwent MRI exams prior to the start of neoadjuvant adriamycin/cytosin (AC) chemotherapy (MRI1), following one cycle of AC (MRI2) and at the completion of chemotherapy, prior to surgery (MRI3). After review of MRI cases with initial disease extended over less than 2 quadrants, this study included 42 neoadjuvant patients (mean age 48.56 years [range 29.7–71.5]). Median follow-up was 178 weeks. Pathology and radiology reports were available for all patients. Total follow-up was 3 years. We applied several modifications to image analysis routines that allowed to quantify MRI background tissue (or "normal tissue") enhancement as well as enhancement values at different locations on and around tumors. Magnetic Resonance Signal Enhancement Ratio (SER) values have been histopathologically shown to positively correlate with microvessel density within tumor. In this project, we lowered the enhancement threshold necessary to detect MRI tumor-enhancing regions, to ensure that slow or nonenhancing tissue regions could be analyzed. For all patients, tumor morphologic patterns, or "imaging phenotypes" were categorized according to the degree of tumor containment from 1, denoting well-circumscribed uni-centric masses to 5, corresponding to infiltrative tumors with ill-defined borders. Tumor volumes were quantified from initial enhancement dynamic data on both MRI1 (baseline) and MRI3 (before surgery). To quantify the uninvolved breast tissue volume, we used a semi-automated technique that performs the segmentation of breast tissue from fat regions without user interaction, therefore avoiding threshold decision issues.

Results: A total of 10 patients recurred. We found that non-tumor mean SER did not change significantly with one cycle of chemotherapy; however, the value of non-tumor mean SER after 1 cycle of chemotherapy was an independent predictor of disease-free survival. These results suggest that we may use non-tumor SER to risk stratify patients receiving neoadjuvant chemotherapy. We also observed a strong association between the initial (pre-treatment) tumor morphologic patterns on MRI with treatment outcome. We found that change in non-tumor breast tissue volume after chemotherapy does relate to tumor phenotype ($p < 0.037$) and breast tissue volume before treatment ($p < 0.029$). Change in non-tumor breast tissue volume after

chemotherapy was also predictive of recurrence ($p < 0.06$). However we found that change in uninvolved breast tissue volume with treatment did not relate to change in tumor volume.

Conclusion: In this study, we hypothesized that the imaging phenotype reflects tumor growth patterns that are influenced by the normal host tissue. We found that change in uninvolved breast tissue volume with treatment was correlated with tumor phenotypes and was shown to be predictive of recurrence. These results suggest that the host tissue may provide treatment response information.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0518.

P9-4: BREAST TUMOR pH: DESIGN EVALUATION AND APPLICATION OF NOVEL REPORTER MOLECULES

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University of Texas Southwestern Medical Center at Dallas

Background and Objectives: Many factors impact the efficacy of cytotoxic therapy for breast cancer. Local acidity (pH), and in particular, cellular transmembrane pH gradients can influence the distribution of therapeutic drugs. Historically, no satisfactory methods existed to measure transmembrane pH gradients in vivo. ^{31}P NMR of inorganic phosphate can reveal intracellular pH (pHi), but the chemical shift dispersion is very small and signal overlap is problematic, not to mention being subject to varying extracellular contributions. We have investigated new approaches using ^{19}F magnetic resonance reporter molecules designed to measure both pHi and extracellular pH (pHe) or pHe alone.

Methods: We designed, synthesized, and evaluated fluoropyridoxols, in particular 6-FPOL (6-Fluoropyridoxine, a vitamin B6 analogue) and derivatives incorporating a trifluoromethyl group to enhance signal to noise (6-(trifluoromethyl)pyridoxine- CF_3POL). Reporter molecules and sodium trifluoroacetate chemical shift reference were infused into Fisher rats bearing 13762NF tumors and ^{19}F NMR was performed at 4.7 T. pH was also assessed using microelectrodes.

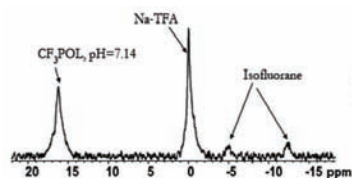


Figure Left: 188 MHz ^{19}F NMR spectrum of 13762NF breast tumor following infusion of pH reporter.

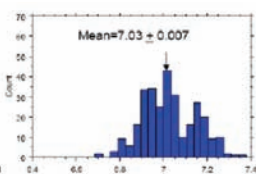


Figure Right: Distribution of pH values measured in a group of 13762 NF rat breast tumor using 6-FPOL.

Results: 6-FPOL provides a large chemical shift range of 9.8 ppm with $\text{pK}_a = 8.2$. In whole rabbit blood and Langendorf perfused hearts, transmembrane pH gradients are observed, but the molecule appears not to enter most tumor cells. CF_3POL has a more satisfactory $\text{pK}_a = 6.82$, though the chemical shift response to acid-base titration is only 1.65 ppm. CF_3POL is specifically restricted to the extracellular compartment in whole blood, perfused heart, and tumors. It was detectable in 8 min following injection of 320 mg/kg and 200 mg/kg NaTFA solution i.p. into Fisher 344 rat. The signal persisted with clearance over a period of about 100 mins. The chemical shift of 16.23 ppm indicated $\text{pHe} = 7.14$. This is in line with electrode measurements in the same tumor type.

Conclusions: Fluorinated vitamin B6 analogs show promise as pH indicators for breast tumors. CF_3POL has an appropriate pK_a and specifically reports on extracellular pH. However, tumors are typically heterogeneous and an imaging method may be required for effective characterization of tumors. These studies were further developed under DAMD 17-03-1-0343-01, where 6-FPOL could be incorporated into β -gal sensitive gene reporters generating a multiparameter reporter- pHe and β -gal.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9381 and National Cancer Institute (U24 CA126608).

P9-5: BREAST CANCER GENE THERAPY: DEVELOPMENT OF NOVEL NON-INVASIVE MAGNETIC RESONANCE ASSAY TO OPTIMIZE EFFICACY

Ralph P. Mason,¹ Vikram D. Kodibagkar,¹ Li Liu,¹ Jian Xin Yu,¹ Weina Cui,¹ Angelina Contero,¹ and Stephen L. Brown²

¹University of Texas Southwestern Medical Center at Dallas and ²Henry Ford Health System

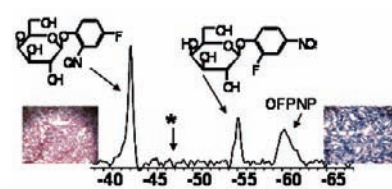
Gene therapy is promising for breast cancer, but implementation is hampered by difficulties assessing transfection and the longevity of gene expression. Thus, there is

increasing interest in the development of noninvasive in vivo reporter techniques to assay gene expression. Historically, the *lacZ* gene encoding β -gal has been widely used and remains exceedingly popular. Noting the broad substrate specificity of β -gal, we developed series of fluorinated substrates as reporter molecules. ^{19}F NMR has several virtues: high magnetogyric ratio, 100% natural abundance, large chemical shift dispersion, and no background signal.

Series of reporter molecules for detection of β -gal activity based on the 19F chemical shift change accompanying enzyme activated cleavage were designed, synthesized, and evaluated in vitro and in vivo. The most effective agents *p*-fluoro-*o*-nitrophenyl- β -D-galactopyranoside (PFONPG) and *o*-fluoro-*p*-nitrophenyl- β -D-galactopyranoside (OFFNPG) are excellent substrates exhibiting a single ^{19}F NMR signal with a $\Delta\delta$ of 6 to 10 ppm upon β -gal catalyzed cleavage. These agents were evaluated in tandem by injecting PFONPG and OFFNPG directly into separated MCF7-*lacZ* and MCF7-wild-type tumors in mice. ^{19}F NMR spectroscopy was used to observe both agents simultaneously, and spatial discrimination between the two tumors was achieved by using separate agents in each tumor.

Using the spectrally resolved reporters PFONPG and OFFNPG two tumors could be interrogated simultaneously. Most significantly, hydrolytic activity observed by ^{19}F NMR corresponded with differential activity in *lacZ*-expressing tumors. There was 100% negative specificity (WT tumors never showed conversion of ^{19}F substrates). For *lacZ*-tumors most measurements showed appearance of product aglycone and well verified by histology using X-gal staining (blue in figure). Unexpectedly, some *lacZ*-tumors showed no ^{19}F NMR activity, and histology revealed little or no blue stain.

The current approach reveals relative expression in stably transfected tumor xenografts, but ultimate application will require in situ transfection where extensive heterogeneity of cellular expression is expected. Thus, imaging will be helpful, and preliminary results show β -gal activity in tumors by ^1H MRI using S-gal together with ferric ammonium citrate. ^1H MRI offers prodigious signal, but detection of transgene activity depends on contrast-to-noise, which may be difficult to interpret in highly heterogeneous tissues. ^{19}F NMR has a much lower effective SNR, but the lack of background signal aids interpretation; thus, both approaches have merit and we continue to develop the methods and applications.



^{19}F NMR of OFFNPG injected into MCF7-*lacZ* tumor with release of product aglycone. No conversion was observed (*) for PFONPG in WT tumor.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0343 and National Cancer Institute (R21 CA120774 and U24 CA126608).

P9-6: DYNAMIC IN VIVO IMAGING OF BREAST TUMORS ENHANCES THERAPEUTIC RESPONSE TO COMBRETASTATIN A4 PHOSPHATE

Dawen Zhao, Karen Chang, Edmond Richer, Nikolai Slavine, Peter Antich, and Ralph P. Mason

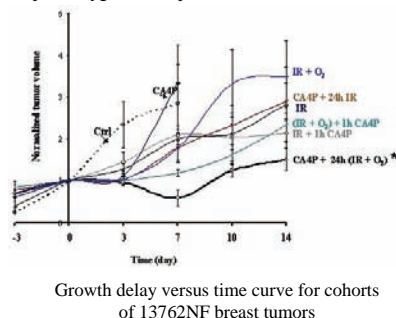
University of Texas Southwestern Medical Center at Dallas

The vascular disrupting agent (VDA), Combretastatin A-4-phosphate (CA4P) causes tumor vascular shutdown inducing massive cell death. We have applied magnetic resonance imaging (MRI) and bioluminescent imaging (BLI) to evaluate pathophysiological response to CA4P in breast tumors. Although massive necrosis can be induced by CA4P, tumors usually regrow from a thin viable rim. Thus, a combination of VDAs with additional conventional therapeutic approaches, e.g., radiation, will be required. Based on imaging data of temporal changes in tumor perfusion and especially hypoxia, induced by CA4P, we have enhanced the combined treatment with radiation in a breast tumor model.

For BLI study of acute vascular perfusion change, MDA-MB 231 human breast tumors were infected to stably express firefly luciferase and highly expressing clones isolated and implanted subcutaneously in the thigh of a nude mouse. CA4P (120 mg/kg; OXIGENE, Inc. Waltham, MA) was injected ip (intraperitoneally) immediately after baseline BLI and then 2 h and 24 h later the BLI time course was repeated. In the CA4P treated group, the detected light emission decreased between 50% and 90% and time to maximum was significantly delayed. Twenty-four hours later, there was some recovery of light emission. Comparable vascular changes were evidenced by dynamic contrast enhance (DCE) ^1H MRI, and further validated by histological study. Tumor hypoxia induced by CA4P in syngeneic 13762NF rat breast tumors was evaluated by using the ^{19}F NMR oximetry approach, FREDOM (Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping). Tumor pO_2 was found to decline within 60 min, become significantly lower at 90 min, and decreased further at 2 h after CA4P infusion. At this time there was no response to breathing O_2 . Some regional recovery was seen 24 h later, but the pO_2 was still signif-

icantly lower than the pretreatment level. Oxygen breathing at the 24 h point modulated tumor pO₂ significantly, which resulted in elimination of tumor hypoxia. Thus, a combination treatment with radiation plus oxygen 24 h post CA4P was tested and compared with alternatives. As shown in the figure, the optimized combination scheme led to a significantly slower growth rate than any other group ($p < 0.05$).

Both BLI and MRI revealed tumor vascular shutdown after CA4P treatment. The quantitative pO₂ values measured by FREDOM provide the potential for exploiting synergy with other oxygen-dependent therapies.



This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0363; National Cancer Institute (U24 CA126608); and National Institutes of Health (P41 RR02584).

P9-7: MAGNETIC RESONANCE SPECTROSCOPY IN BREAST DISEASE

Sandra Brennan, Sunitha Thakur, Wei Huang, Elizabeth Morris, Laura Liberman, Andrea Abramson, Jennifer Kaplan, Anuradha Khilnani, Ralph Wynn, David D. Dershaw, and Hedvig Hricak
Memorial Sloan-Kettering Cancer Center

Introduction: Magnetic resonance imaging (MRI) is playing an increasingly important role in the clinical setting. In 2004, the American College of Radiology (ACR) Committee on Standards and Guidelines published a document with the indications for breast MRI. The American Cancer Society (ACS) revised the recommendations for high-risk screening, recommending annual screening MRI examinations for certain high-risk patients who have at least a 20%–25% lifetime risk of developing breast cancer. The concern is that due to the relatively low specificity of MRI this may generate a large number of false-positive biopsies. Proton MR spectroscopy (1H MRS) may be a useful adjunct to breast MRI reducing the number of benign biopsies without missing the diagnosis of breast cancer.

Purpose: The purpose of this study was to prospectively evaluate the diagnostic performance of MRS in patients with a suspicious mass or biopsy proven cancer on MRI. We correlated the results with pathology to determine the sensitivity and specificity of MRS in the diagnosis of breast cancer.

Materials and Methods: After institutional review board approval (IRB) and informed consent were obtained, we performed breast MRS on 59 patients. All patients had a suspicious mass or biopsy proven cancer 1 cm or larger on MRI. Single-voxel MRS data were collected from a single box volume, which encompassed the lesion with a scanning time of approximately 10 minutes. MRS was defined as positive if the signal-to-noise ratio of the choline resonance peak was greater than or equal to 2. If it was less than 2, it was deemed negative. The MRS findings were then correlated with the pathology results. The 95% confidence intervals (CI) were calculated using the Geigy Scientific Tables.

Results: A total of 59 patients with 60 lesions were imaged. The mean MRI voxel size was 4.6 (range 0.4 to 27.0) cc.

A choline peak was present in 46 of 60 lesions, including 44/48 (92%) cancers and 2 of 12 (17%) benign lesions. Four of the 48 cancers did not demonstrate a choline peak. Susceptibility artifact from localizing clips placed in the lesions at the time of biopsy accounts for 3 of these cases. In the other case, it was due to technical difficulties; the shimming was poor and the lipid peak broad, which may have obscured the choline peak. MRS had a sensitivity of 92% (95% CI, 80%–98%) and specificity of 83% (85% CI, 52%–98%). The positive predictive value of MRS was 96% (95% CI, 85%–99%) and the negative predictive value 71% (95% CI, 42%–92%).

Conclusion: Proton MRS can be successfully incorporated into breast MRI studies for lesions measuring 1 cm or larger, requiring only an additional 10 minutes of scan time. A localizing clip may interfere with detection of the choline peak at MRS. With its high sensitivity and specificity, MRS as an adjunct to breast MRI may decrease the number of biopsies done for benign enhancing lesions.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0568.

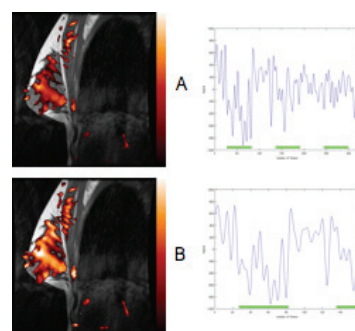
P9-8: BOLD CONTRAST BREAST MRI

Rebecca Rakow-Penner, Laura Pisani, Bruce Daniel, and Gary Glover
Stanford University School of Medicine

Introduction: Blood oxygen level dependent (BOLD) contrast imaging applied to breast tumors may provide useful clinical information on tumor oxygenation. This information has the potential to help guide therapy and diagnosis. Before approaching the study of tumors, our group is interested in better understanding BOLD contrast in healthy breast tissue. We are developing a robust technique for measuring BOLD contrast in the breast.

Methods: A 2D gradient echo spiral pulse sequence with fat saturation and heart saturation was designed for this study. Pure oxygen interleaved with room air delivered through a nasal cannula provided the BOLD stimulus with a block paradigm of 3 periods over 12 minutes. At 3T we developed our protocol and tested our most robust version on the left breasts of four healthy volunteers. A respiratory belt and pulse oximeter were placed on the volunteers to record respiratory motion and cardiac rate. The BOLD signal time series for each voxel was cross-correlated with the periodic stimulus. Sigma and Fourier filters were applied to diminish noise artifact. Coregistration parameters were evaluated to discount motion as causing periodic correlation with the stimulus. The threshold for activation was set at $p = 0.003$.

Results: Figure 1 displays the correlation between the stimulus and the breast tissue of one volunteer. The plots are filtered versions of the time series by frame number in relation to signal intensity of a designated ROI (selected based on activation). The activation maps are superimposed on T1 anatomical images. In two of the four studies, we found an inverse correlation between the stimulus and activation. In the other two volunteers, we found a positive correlation to the stimulus. In A, we found that activation more closely correlated between the first half of the time series but attenuated over the second half. B shows the increased correlation activation map for the first half of the time series.



Discussion: Results at 3T indicate that BOLD contrast in the breast can significantly vary from one person to another. We corrected for motion, along with other controllable parameters, and yet still detected a strong correlation between the stimulus and either a positive or negative BOLD contrast measurement. We believe the results are real and a consequence of either variations in breast vasculature due to menstrual cycle and/or the difference in the fat and water content between volunteers. Future studies will evaluate these potential causes of BOLD contrast fluctuations.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0358 and National Institutes of Health.

P9-9: IN VITRO EVALUATION OF A POLYMERIC CONTRAST AGENT FOR MONITORING BREAST CANCER TARGETED DRUG DELIVERY

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¹University of Maryland, Baltimore, ²University of Maryland School of Pharmacy, and ³University of Utah

Introduction: The use of polymeric conjugates to selectively deliver cytotoxic anticancer drugs to tumor tissues is well established. However, there has not been a concurrent progress in imaging of such drug delivery systems. Previous studies have shown that N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers are suitable carriers for anticancer drugs and magnetic resonance (MR) contrast agents. In this study, we have synthesized, characterized and evaluated HPMA copolymers containing Gd as an imaging agent and Dox as a model drug in vitro.

Experimental Methods: HPMA copolymers with and without Dox were synthesized by free radical precipitation copolymerization of HPMA, methacryloylglycylphenylalanyleucylglycyl-doxorubicin (MA-GFLG-Dox) and aminopropylmethacrylamide-benzyl-1,4,7,10 tetraazacyclododecane-1,4,7,10 tetraacetic acid (APMA-benzyl-

DOTA). Then Gd^{+3} was chelated to the polymeric precursors. The conjugates were characterized for Gd content (Inductively Coupled Plasma Optical Emission Spectroscopy, ICP-OES), T1 relaxivity (1.5 T MRsystem), Dox content (UV spectrophotometry) and molecular weight and molecular weight distribution (size-exclusion chromatography). The stability of the conjugates was determined at different pH values and in the presence of predetermined concentrations (1:1 to 1:125, EDTA:Gd) of EDTA. The toxicity of the conjugates was determined in vitro by MTT assay against a model human breast cancer (MDA-MB-435) and a normal mice fibroblast (NIH/3T3) cell line. The cytotoxicity of Dox containing polymeric conjugates with and without Gd on cancer cells was also evaluated by the same method.

Results: *Relaxivity and stability of conjugates.* Two conjugates, namely HPMA copolymer-Gd and HPMA copolymer-Gd-Dox were synthesized and characterized. The conjugate with Dox exhibited 1.6 times higher relaxivity than the one without Dox, probably due to hydrophobic interactions between Dox molecules that lead to inter- and intramolecular interactions with overall slower local motions and global rotation.

Under acidic conditions (pH=3), 85.8% of Gd remained chelated after 5 days, demonstrating high kinetic stability of Gd-DOTA chelation. Competitive challenge studies

in the presence of EDTA, showed increasing Gd decomplexation from the polymeric conjugate with increasing EDTA concentration.

Cytotoxicity. Incubation of P-Gd conjugate (without Dox) with MDA-MB-435 cells showed higher percentage of viable cells compared to a commercially available contrast agent Magnevist (Gd-DTPA) ($p < 0.019$) at concentrations between 1 and 100 μM equivalent of Gd. The same conjugate showed no significant toxicity on healthy fibroblast cells (NIH/3T3). There is no significant difference in toxicity of polymer-drug conjugates in the presence (P-(DOTA-Gd)-Dox) and absence of Gd (P-DOTA-Dox) suggesting Gd does not interfere with the effect of Dox.

Conclusion: HPMA copolymer – Gd – Dox conjugates were synthesized and characterized. The conjugates were stable and showed higher relaxivity than those without the drug. These systems have potential for monitoring the in vivo fate of the drug delivery system and correlation of localization with efficacy in breast cancer treatment.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0341 and National Institutes of Health (R01 EB020717).

FUNCTIONAL IMAGING

Poster Session P10

P10-1: DEVELOPMENT OF INDOCYANINE GREEN ENCAPSULATED MICROBUBBLES FOR DYNAMIC IMAGING OF BREAST CANCER

Ronald Xiaorong Xu
Ohio State University

PLGA and Indocyanine green (ICG) are nontoxic, biocompatible, and biodegradable agents approved by the U.S. Food and Drug Administration for human use. PLGA microbubbles have been used for ultrasound contrast enhancement and for targeted drug delivery in cancer research. ICG has been used as a contrast agent for near-infrared diffuse optical imaging of breast cancer. However, the amphiphilic property and the aggregation effect of ICG contribute to the complicity of its absorption and emission spectra and place the significant challenge in quantitative imaging applications. In this regard, we aim at developing ICG-encapsulated PLGA microbubbles for enhanced fluorescence stability and concurrent sensitivity to both ultrasound and near-infrared light.

PLGA microbubbles encapsulating ICG were fabricated by an improved double emulsion-solvent method. The size distribution and the fluorescence emission of ICG-encapsulated microbubbles were characterized by flow cytometry and fluorescence microscopy, respectively. Absorption and emission spectra of ICG-encapsulated microbubbles were measured at different concentrations and were compared with those of free ICG in both aqueous solution and human serum albumin (HSA). The potential of ICG-encapsulated microbubbles for breast cancer imaging was demonstrated through a benchtop test on a tissue-simulating phantom using the integrated dynamic near-infrared/ultrasound imaging system recently developed in our lab.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0646 and Wallace Coulter Foundation.

P10-2: NONINVASIVE DETECTION OF ENHANCED PERMEABILITY AND RETENTION OF POLYMERS IN TUMORS

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Anticancer drugs used in chemotherapy are systemic antiproliferative agents (cytotoxins) that preferentially kill the dividing cells. These anticancer agents show poor tumor targeting and hence their use results in severe toxicity to normal cells. Development of strategies with improved targeting of contrast agents and/or drugs to tumor tissues are needed to enhance our capability to image and treat cancer. The tumor vascular linings are made of poorly aligned endothelial cells with large fenestrations, lacking smooth muscle layer, with relatively wide lumen, impaired receptor function, dilated and leaky blood vessels. These abnormalities allow extensive leakage of blood plasma components such as macromolecules, nanoparticles, and lipidic particles into the tumor tissues. The macromolecules are retained in the tumors due to the impaired lymphatic clearance in tumor tissues. This condition is recognized as enhanced permeability and retention (EPR) and it has been extensively used to target nanosized polymeric anticancer agents selectively to tumors. The present work aims to utilize the EPR effect to target fluorescent agent to the tumor tissues and utilize it for detecting breast cancer with high accuracy and minimal discomfort to the patient.

The present work involves establishing a noninvasive procedure to measure the EPR effect in animal model. Balb/c mice (8-week old female) were obtained and inoculated subcutaneously with 4T1 cells (10⁷/mouse) into the dorsal area. We then investigated the EPR effect using PEG [poly(ethylene glycol)] polymers of different sizes as carriers to deliver fluorescent reporter groups to the tumors. PEG was selected as the carrier because it is biocompatible, it is commercially available in various forms for conjugation reactions, and it can be prepared in different sizes for optimizing the EPR properties. Fluorescein-labeled PEG polymers (MW ~10, 20 and 40 kDa) were prepared by reacting thiol-containing polymer of appropriate size with fluorescein-5-maleimide in aqueous sodium-phosphate buffer containing EDTA. The products were purified by size-exclusion chromatography. The purity was determined by gel-permeation chromatography and molecular weights were estimated by MALDI-TOF spectrometry. Fluorescein-labeled PEG polymers (20 mg/mL) were injected intravenously on the 8th day after inoculation (average tumor size: 202±64 mg). A fiber optic-based fluorimeter was used to collect in vivo fluorescence spectra from the tumor and the control site present on the skin of live mice. The fluorescence spectra were collected at different time points by using excitation and emission wavelengths of 480 and 520 nm, respectively. Simultaneously, an IVIS camera was also used to obtain the whole body image of mice injected with polymers. The EPR effect was successfully detected by both methods. It was also observed that the EPR effect show size dependence and it becomes more prominent as molecular weight of polymer increases. Thus, it was possible to noninvasively detect and measure the EPR effect of polymers with different molecular weight in live animals.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7288.

P10-3: DETECTING PROTEINS WITH Xe-129 NUCLEAR MAGNETIC RESONANCE (NMR) BIOSENSORS: SOLUTION-, CRYSTAL-, AND CELL-BASED STUDIES

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Xe-129 nuclear magnetic resonance (NMR) biosensors are promising agents for the early detection and diagnosis of breast cancer, but there is no consensus mechanism by which biomolecular interactions perturb Xe-129 NMR chemical shift. To investigate this further, our lab synthesized a water-soluble cryptophane with unprecedented affinity for xenon in both human plasma and buffer, $K_A = 3 \times 10^4 \text{ M}^{-1}$. This cryptophane was targeted to human carbonic anhydrase (HCA) by attaching a benzenesulfonamide recognition motif via a variable length methylene spacer, $n = 0-2$ units. Dissociation constants ($K_D = 30-120 \text{ nM}$) for these Xe-129 biosensors were obtained by isothermal calorimetry (ITC) for two different isozymes, HCA I and II. Competition binding assays with dansylamide and acetazolamide confirmed binding of the biosensors at the enzyme active site. Biosensor:HCA complex formation produced a significant change in the hyperpolarized Xe-129 NMR chemical shift, $\Delta\delta (\text{Xe-129}) = 5.0-6.5 \text{ ppm}$. This change was much larger than had been observed previously with biotinylated xenon biosensors bound to streptavidin. In HCA, isozyme-specific Xe-129 NMR chemical shifts were observed, with $\Delta\delta (\text{Xe-129})$ of 0.5 ppm differentiating Xe-129@HCA I and Xe-129@HCA II. To gain a better molecular understanding of these Xe-129 NMR results, several biosensor@HCA complexes were crystallized, and structure determinations are under way. In parallel with these studies, our lab generated an RGD peptide-functionalized cryptophane to target cells overexpressing surface integrin receptors. Studies employing fluorescence microscopy confirmed that this compound is nontoxic and is readily taken up by cancer cells by an RGD-mediated mechanism. Cells that do not overexpress integrins (i.e., red blood cells) did not uptake this compound. Efforts are under way to distinguish breast cancer cells from healthy cells using xenon biosensors, based on differences in Xe-129 NMR chemical shift.

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P10-4: RIBOZYME-MEDIATED IMAGING OF RNA AND siRNA IN VIVO

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In vivo imaging of a specific mRNA target has remained a challenge due to the small copy number of mRNA targets per cell. We present a new approach to image mRNA in vivo based on group I introns *Tetrahymena thermophila* ribozyme. Tetrahymena ribozymes constitute a class of catalytic RNA molecules, capable of catalyzing cis- and trans-splicing reactions. We designed plasmid constructs containing the trans-splicing ribozyme, an engineered reporter mRNA, and an antisense sequence against an mRNA target. These constructs were able to bind the mRNA target and produce a fusion mRNA that could be translated into functional reporter enzymes.

Two different reporters have been exploited to image the mRNA of a dominantly negative p53 (p53DN) gene: beta-lactamase for single living cell imaging and firefly luciferase for whole living mice. Analyses of the splicing reaction products confirmed that these constructs were able to target and image mRNA targets in vivo. For a transiently transfected construct, splice products could be seen from the RT-PCR as early as 24 hr after transfection, and splicing-dependent luciferase activity began to increase

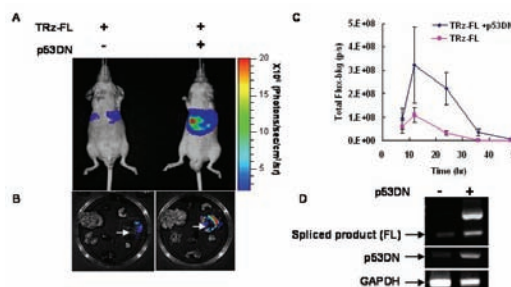


Figure 1. Ribozyme-firefly luciferase reporter for RNA imaging in living mice. (A) Bioluminescent signal in mice 24h after intravenous injection of the ribozyme-luciferase reporter and the p53DN target mRNA or an empty vector. (B) Biodistribution of the luminescent signals in organs dissected out from (A). (C) ROI analysis of bioluminescence plotted against the time in hrs after the injection. (D) RT-PCR results of total RNA isolated from the liver of mice 24 hrs after injection.

at 48 hr and maximized around 72 hr. This reporter can also detect stably expressed p53DN after optimization. A 9.5 ± 1.9 -fold difference was observed between tumors with and without the p53DN mRNA 24 hr following the tumor implant (72 hr after transfection). We have also applied this reporter to image siRNA activity in vivo. The inhibition of target (p53DN) expression by p53 siRNA in cultured cells and in vivo can be imaged directly from the reporter activity, and the reporter signal was dependent on the concentration of siRNA.

In summary, we have shown here the first example of imaging ribozyme-mediated trans-splicing activity and siRNA inhibition in living animals. This splicing-dependent reporter assay may be applied to directly image endogenous mRNAs, especially over-expressed tumor-specific mRNAs in living subjects, and to monitor the siRNA inhibition of target gene expression in vivo.

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P10-5: CATHEPSIN K-SPECIFIC IMAGING OF OSTEOCLASTS AND OSTEOLYSIS

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Metastatic breast cancer cells stimulate local formation and activation of osteoclasts when breast cancer spreads to the skeleton. Bone tissue is typically destroyed in the vicinity of breast cancer metastases by an osteolytic process involving cathepsin K, a cysteine protease strongly expressed by osteoclasts and some types of breast cancer cells. Proteolytic destruction of barriers to cell migration and extracellular matrix facilitates the metastatic process. To detect and study breast cancer bone metastasis in vivo, our approach focuses on near infrared fluorescence (NIRF) imaging of cathepsin K activity with a specific fluorogenic peptide substrate. We have demonstrated cathepsin K-specific cleavage of an autoquenched Cy5.5 synthetic pegylated polymeric substrate that is designed for in vivo imaging. The cathepsin K NIRF substrate probe is highly selective for cathepsin K detection compared to cathepsin B, with a measured selectivity/sensitivity factor of about 20-fold in favor of cathepsin K. A control substrate synthesized with the corresponding D-amino acid α -carbon configuration instead of L-amino acids was poorly cleaved. Single cell NIRF imaging of cathepsin K activity and protein has been accomplished at high resolution in single murine osteoclasts using confocal fluorescence microscopy. These images show that osteoclasts internalize the NIRF cathepsin K substrate by an endocytic process as a prerequisite for intracellular protease cleavage and unquenching of the Cy5.5 fluorescent signal. We have also investigated hypoxia as a regulator of osteoclast formation and activity. Hypoxia has been shown to rapidly increase the osteoclast expression of cathepsin K mRNA (2.2-fold in 1 hr at 5% oxygen v/v). This is relevant because sites of breast cancer metastasis are generally hypoxic. This may lead to greater sensitivity for in vivo imaging of bone metastases because of the dual elevation of osteoclast numbers and cathepsin K activity per cell. Endogenous cathepsin K activity has been quantitated in skeletal tissues of developing mice in vivo using the specific NIRF protease substrate. Ovariectomy of female mice causes a rapid increase in osteoclast activity. This model for locally increasing osteoclast activity was tested to validate NIRF imaging of cathepsin K activity in vivo. After 14 days, but not at 8 days, there is significant reduction of bone mass as measured by the invasive method of bone volume quantitation with micro-computerized tomography. Proximal tibial NIRF quantitation showed that cathepsin K is an earlier marker for osteoclasts, increasing significantly within 8 days after ovariectomy. This increase was blocked by pamidronate administration, consistent with the osteoclast-inhibiting action of this bisphosphonate drug. We conclude that cathepsin K imaging has a superior sensitivity for the endosteal resorptive activity of osteoclasts in the bone marrow compartment. This is promising for the imaging of breast cancer bone metastasis which occurs in this compartment.

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P10-6: USING ENHANCED PERMEABILITY AND RETENTION EFFECT FOR TUMOR TARGETING OF FLUORESCENT AGENT AND SIGNAL AMPLIFICATION

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A major limitation inherent to most of the chemotherapeutic agents is their lack of tumor selectivity. The enhanced permeability and retention (EPR) effect of high molecular weight compounds has been used to target the polymer-linked anticancer agents selectively to tumors. Polymers tend to permeate from the blood vessel and accumulate in tumors owing to the abnormalities in tumor vasculature. They are further retained in the tumor tissues due to the impaired lymphatic clearance. We have con-

firmed that polymers accumulate in tumors depending on their molecular size. Low molecular weight polymers are fast to accumulate in tumors, but they are quickly cleared. High molecular weight polymers, on the other hand, are slow to accumulate in tumors, but they are retained for longer durations. The present work aims to utilize the EPR effect to target fluorescent agent to the tumor tissues in order to detect breast cancer with high accuracy and sensitivity with minimal discomfort to the patient. This will be accomplished by following a three-step simple procedure. Polymer-5'-PNA (peptide nucleic acid) conjugates are administered. The complementary PNA sequence labeled with fluorescein (PNA-5'-Fluorescein) is administered next. By itself, this PNA-fluorescein conjugate would have minimal retention in the tumors because of its smaller size. However, the PNA-Fluorescein conjugates are retained in the tumor tissues due to the Watson-Crick base pairing between the complementary PNA sequences, which noncovalently link the fluorescein group as a polymer-PNA:complementary PNA-Fluorescein complex. The fluorescence signal is detected noninvasively.

The thiol-containing four-arm polymer (PEG-[SH]₄, M. W. = 20,000) was reacted with cross-linker, sulfo-GMBS (N-[γ -maleimidobutyryloxy] sulfo succinimide ester) in 0.1 M sodium phosphate buffer (pH = 7.4). The excess cross-linker was removed by purification through a polyacrylamide-desalting column. The four-arm thiol polymer, now containing the succinimide ester functionality, was reacted in aqueous buffer (pH ~7) with PNA containing the 5'-amino functionality to obtain polymer-(PNA)₄ conjugate. The conjugate was purified by centrifugal ultrafiltration, and the product was characterized by MALDI-TOF spectrometry. The labeled complementary PNA sequence, PNA-5'-Fluorescein, was obtained from a commercial source and characterized by RP HPLC and MALDI-TOF prior to use. The hybridization between complementary sequences was studied by measuring the UV and fluorescence melting curves. The aggregation phenomenon was investigated by spectrophotometric and light-scattering experiments. PNA conjugates with different lengths of PNA sequence have been prepared to take advantage of the relationship between binding affinity and number of complementary base pairs so as to preferentially dissociate the complex in normal tissue and blood, but not in the tumor. In vivo studies are in progress.

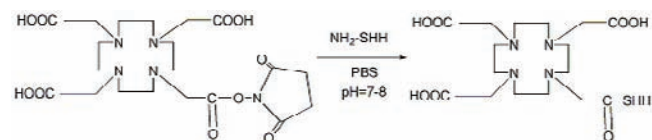
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0342.

P10-7: RADIOLABELED HEDGEHOG LIGAND FOR TARGETED NUCLEAR IMAGING OF BREAST CANCER

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Background: Recently, abnormal activation of classic stem cell signaling pathways, such as the hedgehog signaling pathway, has been observed in breast cancer specimens. Because the hedgehog receptor PTCH is overexpressed in breast cancer cells, radiolabeled sonic hedgehog ligand (SHH) may be a novel imaging agent for the detection of breast cancer and possibly breast cancer "stem cell-like" cells. Therefore, we propose to label SHH with the positron emitter ⁶⁸Ga to assess its usefulness as a potential PET imaging agent for detection of breast cancer.

Methods: DOTA-SHH was prepared by coupling of DOTA-NHS to the 19.5 kDa human N-terminal SHH protein (R&D Systems). DOTA-NHS (1.24 mg, 1.25 μ M) in 2 mL of phosphate buffer, 0.15 mL of 0.1 M DTT, and 0.4 mL of 0.2 M imidazole (pH=8.0) were added to solution of the SSH (0.5 mg, 0.025 μ M) in 1x PBS buffer while on ice. The reaction was carried out at 4°C for 20 hrs. The product was purified and concentrated by ultracentrifugation through filter (MW cut off of 3,000 Da, Amicon) to remove hydrolyzed DOTA. The compound was analyzed by reverse-phased high-performance liquid chromatography RP-HPLC (Vydac, C4 column) using gradient elution: A=H₂O (0.1% TFA, B=CH₃CN (0.1 %TFA) with UV detection at 251 nm/280 nm, (R_T=65 min) and subsequently lyophilized. Product concentration was quantified using Bradford reagent. The resulting conjugate was labeled with ⁶⁸Ga in acetate buffer (pH=4) and heated at 37°C for 30 min. Radiochemical purity and stability were assessed by radio-TLC and radio-HPLC. In vitro bioactivity of ⁶⁸Ga-DOTA-SHH was evaluated using cellular uptake studies in the HH receptor-positive breast cancer cell lines BT-474 and MDA-MB-231. Cells were seeded at a density of 2×10^5 in 6 well plates and grown overnight. Cells were administered 1–2 μ Ci of ⁶⁸Ga-DOTA-SHH and incubated for 15 min to 4 hrs. The cells were washed to remove unbound radioactivity, collected, and counted to determine the amount of specifically bound radioactivity. These data were reported as percent of dose added.



Results: DOTA-SHH was obtained in 45%–60% total yield assessed by HPLC. DOTA-SHH was characterized using MALDI-MS, ESI-MS with purity > 90%. DOTA-SHH was labeled with ⁶⁸Ga with radiochemical purity >97%. HPLC analysis showed the complex was unchanged throughout the labeling reaction and no degradation products were observed. Cellular uptake studies showed increased binding of ⁶⁸Ga-DOTA-SHH to BT-474 and MDA-MB-231 cells from 15 min to 4 hrs. The

uptake ranged from 5.8% at 15 min and reached a maximum value of 7.8% at 4 hrs in MDA-MB-231 cells and 1.7% at 15 min to 4.8% at 4 hrs in BT-474 cells. Uptake correlated with receptor expression and was significantly higher than free ⁶⁸Ga (negative control) in both cell lines.

Conclusions: These preliminary findings suggest that chemical modification and radiolabeling of SHH ligand is feasible. Additionally, our studies show that ⁶⁸Ga-DOTA-SHH maintains high receptor binding in breast cancer cells. Because the expression of the hedgehog receptor is higher in breast cancers than normal breast tissue, noninvasive imaging of the activity of this pathway can be used to detect the presence of breast cancer cells before they are evident by conventional imaging methods.

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P10-8: INTRAVITAL IMAGING IN A TRANSGENIC MOUSE MODEL OF BREAST CANCER AFTER RAPAMYCIN TREATMENT

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Currently there are few ways to complete intravital imaging of well-characterized transgenic mouse models. We are developing an assay to monitor by fluorescence, a transgenic, murine, mammary carcinogenesis model of breast cancer that expresses the well-characterized beta-galactosidase marker. We are utilizing a method to observe the marker in cells and will apply the method to developing lesions and histology preparations without terminating the biologic development of the tumor and hopefully treatment-induced regression to determine efficacy.

We have crossed MMTV WNT 1 mice with mice expressing the β -galactosidase transgene under the control of a β -catenin-responsive promoter (Bat-Gal). These animals have a well-characterized, known developmental pattern or sequence that originates in the mammary fat pad in a precise pattern and sequence and express β -gal in a β -catenin-responsive manner. The β -catenin protein is stabilized in MMTV-Wnt1-induced mammary tumors. Using an approach developed by Tung et al (2004), we are developing methods to image, localize, and quantify the appearance of Wnt1-induced mammary tumors by measuring the activity of β -gal with a probe (DDAOG) that emits a red fluorescent signal upon β -galactosidase-dependent cleavage and appropriate excitation. We have confirmed the fluorescence via flow cytometry and tested the staining in coherent cells, cell pellets, and transgenic mice. Our model uses the Maestro and Nuance instruments (www.cri-inc.com), designed for small animal, multispectral, in vivo fluorescence imaging. We mathematically un-mix the image cubes and separate the DDAO and background components using spectrums deduced from control specimens and saved in a spectral library. We can quantify expression using software programs developed at the Van Andel Research Institute (Kort et al, 2003). The anatomical position of the mouse for breast mammary development can be localized to a specific region using the ventral, lateral, left teat/node. Upon sacrifice, the animals will be further characterized using both the Nuance spectral and Zeiss 510 confocal microscope to localize, quantify, and develop 3D reconstructions of the tumors, treatment effect, and vector controls. In preliminary studies, we have shown that daily injection of rapamycin can inhibit the development of mammary tumors in the MMTV-Wnt1 model. We hope that this work will allow us to efficiently and quickly determine the best doses of rapamycin for treatment and serve as a paradigm for how conceptually similar imaging strategies can be used to measure the efficaciousness of other treatments.

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P10-9: DEVELOPMENT OF IMAGING AGENTS TARGETING THE NONCLASSICAL ESTROGEN RECEPTOR GPR30 IN BREAST CANCER

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The classification of breast cancers based upon the expression of the nuclear estrogen receptor (ER α) correlates with the estrogen-dependency of tumors and represents one of the best prognostic factors due to the availability of antiestrogens such as tamoxifen and more recently the aromatase inhibitors. Estrogen promotes cell proliferation and inhibits apoptosis through a complex signaling cascade resulting in transcriptional changes that may include modulation of tumor suppressor function. A new type of estrogen receptor, GPR30, a transmembrane G protein coupled receptor that is expressed in multiple cancer types was recently identified. This discovery has stimulated interest in the underlying mechanisms involving GPR30 that may be related to estrogen-dependence in cancers and consideration of the potential for GPR30 as a biomarker and diagnostic imaging target for breast cancer. We have developed a series of synthetic compounds that are capable of targeting intracellular estrogen receptors and evaluated their potential as imaging agents using cell-based and animal models. We describe a new class of estrogen derivatives, incorporating a neutral, cell

membrane permeable tridentate Tc-99m-tricarbonyl pyridin-2-yl hydrazine chelate substituted at the 17- α position of estradiol, that exhibit affinity for all classes of estrogen receptors. We have also developed the first GPR30-selective SPECT imaging agents derived from the non-steroidal GPR30 agonist G1, a tetrahydro-3H-cyclopenta[c]quinoline. A series of compounds derived from this scaffold were synthesized, incorporating the pyridin-2-yl hydrazine chelate for radiolabeling with Tc-99m, polyamino-polycarboxylate chelates for In-111, or a tributyltin group for exchange with iodine-125. Radioligand receptor binding studies were performed on ER α / β - and GPR30-expressing human breast cancer MCF-7 cells and GPR30-expressing human endometrial carcinoma Hec50 cells. Blocking studies were performed with the GPR30-specific ligand G1 and estradiol to characterize GPR30 specificity. In vivo biodistribution studies were performed on ovariectomized female athymic mice bearing human breast cancer MCF-7/18 tumors. These first-generation GPR30-targeted agents incorporating different radionuclides are important lead compounds for evaluating this new estrogen receptor as a diagnostic imaging target in breast cancer

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P10-10: TARGETING THE ESTROGEN RECEPTOR WITH METAL CARBONYL DERIVATIVES OF ESTRADIOL

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We have designed and synthesized a series of novel rhenium tricarbonyl derivatives of estradiol as potential breast cancer imaging agent based upon the current understanding of the steroid ligand-estrogen receptor binding process. Although simple 17- α -(hetero)arylvinyl derivatives of estradiol can be prepared via palladium(0) catalyzed Stille coupling reaction, the incorporation of the rhenium tricarbonyl group is more challenging. In this study we evaluated metallated and nonmetallated approaches to the preparation of rhenium tricarbonyl substituted pyridyl vinyl estradiols. The synthesis constitutes the first report of a Stille coupling between a metallated complex and a vinylstannane. The final products retain significant estrogen receptor binding properties suggesting that further structural modifications of the estradiol ligand, known to enhance affinity, may lead to estrogen receptor-selective breast cancer imaging agents.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0544 and National Institutes of Health.

P10-11: BINDING AFFINITY DETERMINATION OF α V β 3 TARGETING LIGANDS FOR IMAGING OF OSTEOLYSIS

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Among breast cancer patients with metastatic disease, up to 80% will have metastatic bone lesions. Nuclear medicine techniques such as bone scans have good sensitivity for detection of osteoblastic bone metastases relative to X-ray, CT, or MRI. However, no currently available nuclear medicine radiopharmaceuticals target osteolytic bone metastases. Integrin $\alpha_v\beta_3$ is upregulated in osteolysis. Here, we investigate bifunctional ligands for binding affinity to $\alpha_v\beta_3$ with good specificity over $\alpha_5\beta_1$ in an isolated competitive binding assay. Bifunctional ligands will allow for targeting with a peptide or peptidomimetic as well as radiolabeling with ⁶⁴Cu via a macrocyclic chelator for Positron Emission Tomography (PET).

For the binding assay, integrin $\alpha_v\beta_3$ or $\alpha_5\beta_1$ was coated onto 96-well plates. Plates were then blocked with BSA. After washing, biotinylated vitronectin with or without serially diluted ligands were allowed to bind to the integrins. Following washing, bound biotinylated vitronectin was detected by adding ExtrAvidin-Alkaline Phosphatase and using *p*-nitrophenyl phosphate as the chromagen. Each concentration data point was done in triplicate, and each binding experiment was performed at least twice. Non-linear regression was used to fit binding curves and calculate IC₅₀ values (Table 1).

	IC ₅₀ (nM)	
	$\alpha_v\beta_3$	$\alpha_5\beta_1$
c(RGDyK)	3.7	171
Cu(II)-CB-TE2A-c(RGDyK)	6.0	194
Cu(II)-TA138	0.020	25.6
KCRGDC	10.4	921
GRGDS	15.9	>5,000
CB-TE2A-E-[c(RGDyK)] ₂	2.91	ND
ND=not determined		

Table 1. IC50 values for integrin binding

A non-peptide $\alpha_v\beta_3$ antagonist, TA138 (provided by Bristol-Myers Squibb), showed better affinity for $\alpha_v\beta_3$ than RGD (Arginine-Glycine-Aspartic acid) peptides, but decreased selectivity over $\alpha_5\beta_1$.

Lactam-cyclized RGD peptides demonstrate slightly better affinity for $\alpha_v\beta_3$ than disulfide-cyclized or linear RGD peptides. Disulfide-cyclized and linear RGD peptides exhibit comparable selectivity against $\alpha_v\beta_3$, better than that of lactam-cyclized peptides. A dimer of c(RGDyK) displayed similar affinity for $\alpha_v\beta_3$ as the monomer. The linear peptide has the best selectivity of the ligands investigated. Conjugation of Cu(II)-CB-TE2A to c(RGDyK) did not affect the binding affinity or selectivity of the peptide.

The ^{64}Cu -labeled lactam-cyclized, disulfide-cyclized and linear peptides will be evaluated further to determine internalization in $\alpha_v\beta_3$ -positive tumor cell lines. The most promising ligand will be used for in vivo imaging with microPET/CT. Improvement in affinity and/or specificity may increase the tumor:background ratios increasing imaging sensitivity. New PET tracers will potentially enable us to image an increase in number of osteoclasts, facilitating earlier lesion detection and monitoring of therapy to osteolytic bone metastases.

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P10-12: NONINVASIVE MONITORING OF BREAST CANCER DURING NEOADJUVANT CHEMOTHERAPY USING OPTICAL TOMOGRAPHY WITH ULTRASOUND LOCALIZATION

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We investigated the feasibility of using optical tomography in the near-infrared spectrum (NIR) combined with ultrasound (US) localization (NIR/US) to monitor tumor vascular changes and pathologic response during chemotherapy for locally advanced breast cancer. Eleven female patients were studied during treatments with a combined imager consisting of a commercially available U.S. system coupled to a NIR imager. Tumor vascular content was assessed based on total hemoglobin (tHb) concentration and volume obtained from NIR data. Percentage blood volume index (PBVI) was calculated as the product of tHb concentration and volume using the pre-treatment tHb image as the baseline. At treatment completion, pathological assessment revealed three response groups: complete or near-complete responders A, partial responders B, and non-responders C. The mean PBVI of groups of A, B, and C at the treatment completion were 29.1%-6.9%, 46.3%-3.7%, and 86.8%-30.1%, respectively (differences highly statistically significant, $p < 0.04$). At the end of cycle two, the PBVI of group A is noticeably lower than the partial ($p = 0.091$) and non-responder groups ($p = 0.075$) with moderate statistical significance. Our findings indicate that NIR/US using PBVI can be used during chemotherapy to monitor tumor vascular changes. It also may distinguish pathologic response during treatment allowing for tailoring therapies to response; this requires more patients to confirm.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4133; National Institutes of Health (R01 EB002136); and Patrick & Catherine Weldon Donaghue Medical Research Foundation.

P10-13: METABOLIC MAPPING IN BREAST CANCER MODELS WITH COMBINED FLUORESCENCE SPECTRAL AND LIFETIME IMAGING MICROSCOPY

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¹University of Wisconsin, Madison and ²Duke University

A tumor is an abnormal growth of tissue due to increased proliferation of cells in a tissue. Because of their elevated proliferation rates, tumor cells have a higher rate of glycolysis than normal cells, which was discovered by Otto Warburg nearly three quarters of a century ago. This "Warburg Effect" is the basis for the positron emission tomography (PET) of tumors used today in the clinic. The mechanisms that link tumor progression and metabolic states are still not fully understood in part because it has been difficult to visualize the dynamics of metabolism in vivo. Thus, there is great interest in noninvasive imaging techniques that would allow one to monitor changes in metabolism between normal and carcinoma cell lines.

Multiphoton laser-scanning microscopy (MPLSM) is a widely used technique in the field of biomedical research to study living samples due to its deep sectioning, improved viability, and signal-to-background ratio as compared to other live cell imaging methods. In this project, we designed and implemented a combined fluorescence spectral and lifetime microscope system (SLIM) to simultaneously collect intensity, excited state lifetime, and emission spectra information of the fluorescence signal. Furthermore, we demonstrated that fluorescence lifetime imaging microscopy (FLIM) has the potential to monitor cellular metabolism in vivo and can be used to look at important intrinsic fluorophores such as flavin adenine dinucleotide (FAD) and the reduced nicotinamide adenine dinucleotide (NADH). By comparing emission spectrum with

SLIM, we could differentiate NADH from FAD and demonstrate that the endogenous fluorescence signal seen at 780 nm excitation is mainly contributed by the reduced form of NADH, one of the important metabolic intermediates.

We investigated possible metabolic differences between human breast malignant cells (T47D and MDA-MB-231) and human breast epithelial cells (MCF10A) and found that in the MCF10A cells the fluorescence lifetime of bound NADH changes in response to microenvironment changes while in the T47D and MDA-MB-231 cells the lifetime stays constant. Our results suggest that lifetime measurements of the cofactor NADH can be used to monitor cellular metabolism and have the potential to differentiate between normal cells and breast cancer cells.

Since NADH may bind to different proteins when metabolism changes as result of microenvironment changes, the ability to not only monitor the bound and free states of NADH but determine which binding proteins are there would be very advantageous. One possible technique that is being investigated is the measurement of polarization anisotropy decays. There is some evidence that suggests that combining polarization measurements with spectral and lifetime collection may reveal information about binding states and partners for NADH.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0397 and National Institutes of Health.

P10-14: RESPIRATORY CHALLENGES: A NEW PROGNOSTIC METHOD TO MONITOR THE EARLY EFFECTS OF CANCER THERAPY

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Respiratory challenges such as hyperoxic gas intervention have been used to increase tumor oxygenation so that the efficiency of cancer treatments can be improved. However, in this study, we applied respiratory challenges to monitor tumor responses to the therapies including conventional chemotherapy, vascular targeting chemotherapy, and radiation therapy by measuring tumor oxyhemoglobin changes ($\Delta[\text{HbO}_2]$) with a continuous wave near infrared spectroscopy (CWNIRS).

We have applied a conventional chemotherapeutic agent, cyclophosphamide (CTX), to two groups of rats bearing syngeneic 13762NF mammary adenocarcinomas: one group ($n=5$) received a single high dose ip (200 mg/kg CTX) while the other group ($n=3$) was treated with continuous low doses (20 mg/kg CTX ip for 10 days). We have also monitored the effects of combretastatin A4 phosphate (CA4P, 30 mg/kg, ip), which is one of the vascular acting agent from another group of rats ($n=10$). For the radiation treatment, rats were divided into 2 groups: Group 1 ($n=3$) breathed air and Group 2 ($n=3$) breathed oxygen during 10 min of irradiation (30Gy, 3Gy/min). Time

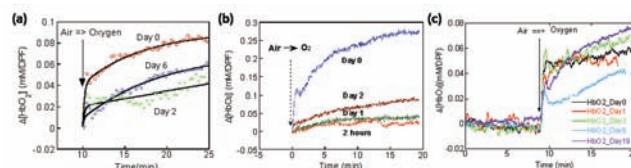


Figure 1 Changes of [HbO₂] increase during oxygen intervention while tumors are under (a) low dose CTX treatment, (b) CA4P treatment, and (c) radiation treatment.

courses of changes in tumor $\Delta[\text{HbO}_2]$ were measured by CWNIRS on tumors non-invasively with an inhaled gas sequence of air-oxygen-air before and after treatment. After CTX treatment, significant changes in vascular hemodynamic response to oxygen inhalation were observed from both a single high dose and metronomic low doses groups. Fitted parameters from $\Delta[\text{HbO}_2]$ increase during oxygen intervention were correlated well with the tumor size regression. CA4P administration caused significant decreases in tumor oxygenation by blocking the blood flow into tumors, but tumor vasculature recovered its function slowly at day 1 after CA4P administration. We found that oxygen inhalation during radiotherapy may enhance the efficacy of radiation treatment by providing more oxygen in poorly perfused region of tumors. However, we did not achieve statistically significant differences of radiation therapy efficacy between two groups due to the small number of animals.

These results suggest that the effect of cancer therapy in the tumor could be monitored by a noninvasive NIRS from detecting the changes of tumor hemodynamics induced by respiratory challenges. Thus, respiratory challenges may be used for the diagnosis/prognosis of tumors and be helpful to optimize individual treatment plan.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0353 and National Cancer Institute (P20 CA086354).

NANOTECHNOLOGY I

Poster Session P11

P11-1: IDENTIFICATION OF TYPE I IGF RECEPTOR EXPRESSION BY ANTIBODY-CONJUGATED QUANTUM DOTS IN BREAST CANCER

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The type I insulin-like growth factor (IGF) receptor (IGF1R) is a transmembrane receptor tyrosine kinase involved in breast cancer proliferation, survival, and metastasis. Several monoclonal antibodies directed against the receptor are in clinical trial. To develop a methodology to detect and measure IGF1R levels in breast cancer cells, we covalently conjugated an IGF1R antibody, AVE-1642, with quantum dots (Qdots), which are nanocrystals that emit fluorescence upon excitation. AVE-1642 Qdots only bound to cells that express IGF1R, and measured IGF1R levels by fluorescence emission. After binding to the cell surface, AVE-1642 Qdots underwent receptor-mediated endocytosis, localized to endosome, and later translocated into the nucleus. Treating MCF-7 cells with AVE-1642 Qdots, but not pure Qdots, downregulated IGF1R levels and rendered cells refractory to IGF-I stimulation. Furthermore, cell proliferation was slightly inhibited by AVE-1642 Qdots, but not the pure Qdots. Our data suggest that AVE-1642 Qdots can be used to detect IGF1R expression and measure changes of cell surface receptor levels in vitro in breast cancer cells. In addition, after tail vein administration, AVE-1642 Qdots has the ability to target xenograft breast tumors expressing IGF1R in nude mice, with some non-specific uptake at the reticuloendothelial system in the liver, spleen, and bone marrow.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0499.

P11-2: RADIATION DOSE ENHANCEMENTS IN GOLD NANOPARTICLE SOLUTIONS: A MONTE CARLO SIMULATION WITH NANOPARTICLE GEOMETRY

Sean Zhang,¹ Junfang Gao,¹ Zhonglu Wang,¹ Mohammad R. Salehpour,¹ Rebekah A. Drezek,² and Tse-Kuan Yu¹

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Purpose: Previous lab studies suggest biological effective dose enhancement due to gold nanoparticles in radiation therapy. The purpose of this study was to investigate physical dose enhancement in gold nanoparticle water solutions with nanoparticle geometry.

Methods and Materials: We simulated an Ir-192 brachytherapy source in a water phantom with Monte Carlo Geant4 code. Our code was first benchmarked with previously published data. After validating the code, we modeled gold nanoparticle water solution with up to 10^{13} gold nanospheres per cc and examined their dose enhancement effect. For comparison, we also implemented a gold-water mixture model used in other studies, in which gold concentration was used to present gold nanoparticles as a whole without nanoparticle geometry (Figure 1).

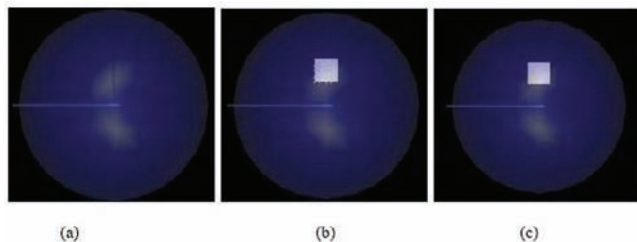


Figure 1. Monte Carlo simulation animation view of (a) the water phantom case, (b) the gold nanosphere case, and (c) the gold-water mixture case. Case (b) was described in Figure 2. In case (c), gold nanospheres were replaced by the same concentration and volume of the gold-water mixture, while the location of the region was kept the same as in case (b).

Results: In our benchmark test, our calculated dose-rate constant, radial dose function $g(r)$, and 2D anisotropy function were in agreement within 2% of those reported previously. We found that radiation dose was enhanced up to 60% in water phantom with 10^{13} gold nanospheres per cc, irradiated by the Ir-192 brachytherapy source. In our comparison study, dose enhancement was overestimated up to 16% in the gold-water mixture model than what we found in our nanoparticle model (Figure 2).

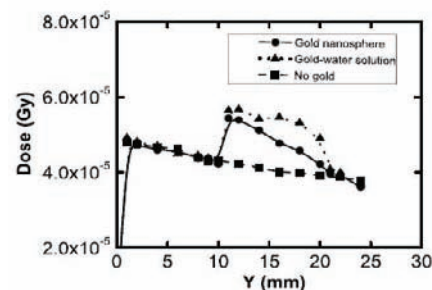


Figure 2. Absorbed dose distributions when no gold was present and when gold nanospheres or gold-water mixture was present. A Parallel photon beam of energy of 380keV was modeled as emanating from the Ir-192 HDR brachytherapy source. Dots, squares, and triangles show actual calculation points. Dashed line with squares shows the dose distribution in the no-gold case; the maximum uncertainty in the data was 1.1%. Solid line with dots shows the dose distribution when the gold nanosphere solution was present, with a maximum uncertainty of 1.9%. Dotted line with triangles shows the dose distribution when the gold-water mixture was present, yielding a maximum uncertainty of 1.2%.

Conclusions: We have built, what we believe for the first time, a large number of gold nanoparticles in nanometer-scale geometry using the Geant4 Monte Carlo simulation code. Thus, we were able to estimate the effect of dose enhancement due to gold nanoparticles more realistically and accurately. With this model we can study various radiation dose parameters, such as radiation energy and nanoparticle geometry and concentration, to optimize the radiation dose enhancement effect, which, in turn, will provide valuable guidelines for future lab studies and clinical applications.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0672.

P11-3: INTEGRATED MOLECULAR TARGETING OF IGF1R AND HER2 SURFACE RECEPTORS AND SELECTIVE DESTRUCTION OF BREAST CANCER CELLS USING CARBON NANOTUBES

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Molecular targeting and photodynamic therapy have shown great potential for selective cancer therapy. We hypothesized that monoclonal antibodies that are specific to the IGF1 receptor and HER2 cell surface antigens could be bound to single wall carbon nanotubes (SWCNT) in order to concentrate SWCNT on breast cancer cells for specific near-infrared phototherapy. SWCNT functionalized with HER2 and IGF1R specific antibodies showed selective attachment to breast cancer cells compared to SWCNT functionalized with non-specific antibodies. After the complexes were attached to specific cancer cells, SWCNT were excited by ~808 nm infrared photons at ~800 mW cm⁻² for 3 min. Viability after phototherapy was determined by Trypan blue exclusion. Cells incubated with SWCNT/nonspecific antibody hybrids were still alive after photo-thermal treatment due to the lack of SWCNT binding to the cell membrane. All cancerous cells treated with IGF1R and HER2 specific antibody/SWCNT hybrids and receiving infrared photons showed cell death after the laser excitation. Quantitative analysis demonstrated that all the cells treated with SWCNT/IGF1R and HER2 specific antibody complex were completely destroyed, while more than 80% of the cells with SWCNT/nonspecific antibody hybrids remained alive. Following multi-component targeting of IGF1R and HER2 surface receptors, integrated photo-thermal therapy in breast cancer cells led to the complete and selective destruction of breast cancer cells. These results show that by targeting surface receptors and NIR dosing, one can achieve a high degree of efficacy in destruction of cancer cells. While past studies have shown the utility of nanotubes to transport DNA and act as NIR agents to kill cells, this is the first study to show the multicomponent targeting of more than one surface receptor (IGF1R and HER2) using SWCNT and simultaneous photo-dynamic therapy due to their high optical absorbance. The energy used in the destruction of cancer cells can be estimated to be ~200 nW per cell, too low to create any damage to the normal cells compared to past nanoparticle and nanotube-based cell-killing techniques. Past nanoparticle and nanotube-based cell-killing techniques have used 4-35W cm⁻² and 1.4-3 W cm⁻² for 3-4 min producing temperature changes for cell killing. Our method uses half the laser power of past nanotube-based cell-killing techniques and a 5-44 times lower laser power compared to past nanoparticle technologies. High internalization of nanotubes into the cells therefore minimizes the amount of energy necessary for killing the cells. Past nanotube studies have targeted the folate receptor that is overexpressed in folate-positive cancer cells and nanoshells have targeted more breast cancer relevant HER2 surface receptors. We have taken

this a step further by showing multicomponent targeting integrated with photo-dynamic therapy. By using nanostructures as biological transport carriers to target more than 1 surface marker, one can increase the efficiency of therapeutics that can lead to higher selectivity. Multicomponent targeting and internalization followed by photo-thermal cell killing can therefore improve the efficiency and selectivity of therapeutics.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0668 and National Institutes of Health IDeA Networks of Biomedical Research Excellence.

P11-4: LHRH AND DOXORUBICIN CONJUGATED GOLD NANOPARTICLES FOR BREAST CANCER TREATMENT

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University of Louisville

Background and Objectives: Breast cancer remains the most common cancer among women, accounting for 212,940 new cases and 40,970 deaths in 2006 alone. The primary treatment for cancer is cytoreductive surgery followed by adjuvant chemotherapy, radiotherapy, or both. While this method is successful in the majority of cases, it is accompanied by cytotoxicity to normal cells and organs. The purpose of this study is to develop a method to treat breast cancer more specifically and to minimize the systemic toxicity. Direct targeting of cancer cells can be achieved by using agents specifically directed to binding sites on cancer cells such as carbohydrates, lectins, surface proteins and receptors, etc. LHRH receptors are present in hormone-related tumors including breast cancer. In normal tissues, LHRH receptors are not expressed or expressed at an undetectable level. By coupling these receptors to nanoparticles, breast cancer tumors and their metastases can be targeted directly. We hypothesize that targeting the LHRH receptor using nanoparticles carrying the LHRH analog and cytotoxic agent (doxorubicin) would enhance drug uptake by cancer cells with reduced toxicity to normal cells.

Methods: To test our hypothesis, we conjugated gold nanoparticles (20–30 nm) with LHRH analog [D-Trp⁶]LHRH and tested for its binding to the LHRH receptor and activation of receptor by performing ligand binding assays and production of intracellular cAMP. The effect of LHRH and doxorubicin conjugated to gold particles on breast tumor cell growth was performed using cell proliferation assays. Distribution of LHRH conjugated particles in tumor, metastatic cells, and other tissues was determined by injection of fluoro-LHRH conjugated gold particles in nude mice bearing sc breast tumors followed by examination of tumors and tissues under fluorescence microscope.

Results: Our results showed that conjugation of gold nanoparticles with [D-Trp⁶]LHRH and doxorubicin retained its binding affinity for and biological activation of the LHRH receptor. In our studies, we showed that treatment of pituitary gonadotrope tumor cell line LBT2 that express high levels of high affinity LHRH receptors resulted in 87% cell death and loss of cell viability within 48 h at a concentration of 2.5 or 5 nM of conjugated doxorubicin. Similarly, treatment of breast tumor cells "MCF-7" with LHRH and doxorubicin conjugated gold particles showed a significant inhibition of cell growth. Whereas no change in cell proliferation or cell apoptosis was observed in cells treated with equal amounts of [D-Trp⁶]LHRH or free doxorubicin. The amount of conjugated doxorubicin required to achieve same level of cell death was found to be 100- to 200-fold lower than the free doxorubicin. Injection of LHRH conjugated fluoro-gold particles (alexa 594 labeled gold particles) into nude mice bearing sc tumors and metastatic lung cancer resulted in high accumulation of particles in tumors and metastatic cells in lung. A small amount of particles was observed in pituitary or liver. In contrast unconjugated particles showed high accumulation in liver and very low levels in tumors and lungs.

Conclusions: Results obtained from our studies are very exciting and suggest that [D-Trp⁶]LHRH can be specifically used to target breast cancer cells to deliver anti-cancer agents to induce cell death.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0662.

P11-5: SURFACE FUNCTIONALIZED NANOPARTICLES FOR PROXIMITY-ACTIVATED DETECTION AND IMAGING OF BREAST CANCER

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Vanderbilt University

Site-directed therapy of metastatic and primary breast cancer promises to minimize systemic toxicity associated with chemotherapy agents that have no specificity for pathologic tissues. We have developed a multifunctionalized nanoparticle specifically designed to target breast cancer, including metastases.

Quantum dots (QDs) were functionalized with a matrixin (MMP-7) cleavable, proximity-activated substrate, PEG-peptide-PEG (PA construct), where cleavage occurs at the peptide bridge. The QDs were further functionalized with folic acid, a strong breast cancer cell targeting ligand with well-known binding to some normal tissues. The construct initially conceals the folate ligand and reduces binding to non-target tissues. MMP-7 activated cleavage of the PA construct and subsequent unmasking of the folate ligand occurs only at the site of the breast tumor, resulting in a proximity-activated targeting (PAT) system. The activity of QDs functionalized with both the PA construct and folic acid (FA-QD-PA) was compared to QDs functionalized with only folic acid (QD-FA).

Cellular association of QD-FA nanoparticles with the human breast cancer cell line MDA-MB-231 revealed that $41.9 \pm 6.5\%$ of the cells were fluorescently labeled. Addition of the PA construct reduced QD-FA recognition by MDA-MB-231 cells to $2.1 \pm 0.4\%$. MMP-7 cleavage of the protected FA-QD-PA construct restored MDA-MB-231 recognition to $21.9 \pm 5.4\%$, a significant increase compared to the uncleaved multifunctional nanoparticle construct (one-way ANOVA, $p < 0.001$, $n = 3$). This in vitro data suggests that the PAT nanoparticle model could function effectively in vivo for specific targeting of breast cancer and metastasis. In the next phase of research, the ability of the FA-QD-PA and FA-QD nanoparticles to detect breast cancer in vivo will be tested using murine models of human breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0306.

P11-6: IDENTIFYING PROTEIN-PROTEIN INTERACTIONS AND DYNAMICS IN BREAST CANCER BY NANOCHANNEL DEVICE

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Breast cancer is the most common type of cancer among woman in this country and deregulations of signal pathways are often responsible for the cancer progression. Many mysteries of cancer-related signal pathways have been revealed by current molecular biology research and the detection of protein-protein interactions is one of the critical techniques. The general techniques for protein-protein interactions detection include immunoprecipitation, immunofluorescence staining, and fluorescence resonant energy transfer. These techniques can detect specific protein in cultured cell and in tissue and have been applied in most biomedical research. However, all of them have various limitations. For instance, only two molecules could be examined in one experiment and the detection usually requires long processing time with complicated procedures and large sample amount. Moreover, the detection of signals currently is still relied on X-ray film exposure or eye-direct observation that may limit the sensitivity and accuracy. We are developing a nanochannel detection system to avoid these defects and to provide rapid and accurate detection in breast cancer signal transduction research. The principle is that nanochannel fluidics could only allow a single protein complex to pass through the detection point of fluorescence spectroscopy during examination and the result from the single protein complex could provide us most direct information of protein-protein interaction. Since the detection level is down to the single protein complex, it could highly increase the detection sensitivity. Currently, we have successful fabricated different size of nanochannels and we recognized that top-down fabrication approach on transparent silicon-based wafers could provide the best quality nanochannels. The channel was then integrated to the fluorescence spectroscopy for single fluorescence detection. To test the performance of the device, fluorescence antibodies were loaded into the channel and we successfully detected a single fluorescence signal during the experiment. Subsequently, we detected the protein complexes harvested from the cultured cell. Two cancer-related proteins, MAX and MAD, which could form heterodimer complex to antagonize the oncoprotein MYC, was first time to be detected in single complex level. Thus, our results suggested that single protein complex detection is feasible and the nanochannel-based protein complex detection could become a novel technique in the future breast cancer signal transduction research.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0709.

P11-7: GENETICALLY ENCODED, TARGETED, AMPLIFIABLE, IMAGING AGENTS FOR THE EARLY DETECTION OF BREAST CANCER

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¹Massachusetts General Hospital and ²Massachusetts Institute of Technology

Early detection and accurate staging of breast cancer are essential for prolonged survival and in choosing effective therapies. Mammography is currently the standard screening tool used clinically for the detection of breast cancer; however, it does not relay important molecular information useful for treatment stratification or provide

tumor stage information. Although some markers such as Her2/neu or estrogen receptor (ER) expression are used clinically to categorize breast cancer, these markers are present only in subsets of cancers and are detected using biopsy specimens collected from invasive procedures such as percutaneous biopsy or sentinel lymph node biopsy. The development of novel molecularly targeted imaging probes would have a number of advantages such as: (1) allowing much earlier detection of lesions based on molecular signatures, (2) providing rational approaches to stratify patients for specific treatments, (3) monitor more reliably for local recurrence and distant metastases, (4) as biomarkers or “surrogates” for new targeted therapies, and (5) to differentiate recurrence from radiation-induced changes. Our overall goal in this work is to identify novel biomarkers and molecular imaging agents for the early detection of breast cancer. Specifically we have (1) used phage display selection on CD44+CD24- breast cancer stem cells to identify lead peptides for the development of targeted imaging agents and (2) engineered new vectors to develop genetically encoded multimodal imaging scaffolds (GEMS) that can be imaged via MRI and optical modalities, which will allow us to test the breast cancer-targeted GEMS in vivo using MRI and optical imaging. Clinical imaging, early detection of breast cancer, as well as treatment stratification of patients with breast cancer utilizing the developed imaging agents may substantially decrease the morbidity and mortality of patients diagnosed with breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0703.

P11-8: NANOPARTICLE CONTRAST AGENTS FOR ENHANCED MICROWAVE IMAGING OF BREAST CANCER

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The critical need for new technologies to improve early detection and diagnosis of breast cancer is widely recognized. During the past decade, narrowband and ultra-wideband microwave imaging has been under intensive investigation as an alternative imaging modality for early-stage breast cancer detection. The goal of our research project is to explore the feasibility of using biocompatible nanoparticles, which can be systemically delivered to the site of the malignancy, to enhance the microwave response of breast tumors. Our research is focused on the investigation and optimization of the microwave response of a variety of nanoparticle contrast agents for enhancing the sensitivity of microwave breast imaging.

Our first specific aim is to experimentally characterize the effective electromagnetic properties of a variety of micro/nanoparticle dispersions at microwave frequencies. We are working with dispersions of several types of particles with various concentrations in dielectric liquid. We are conducting wideband and narrowband microwave frequency measurements of the effective dielectric properties of these dispersions and analyzing measured data to assess dielectric parameter changes due to the presence of the micro/nanoparticles.

Our second aim is to develop theoretical/numerical tools to model microwave interactions with micro/nanoparticles and utilize these tools to design micro/nanoparticles that optimize the microwave contrast of malignant breast tumors. We are theoretically characterizing the effective electromagnetic properties of a variety of micro/nanoparticle dispersions at microwave frequencies using 2D and 3D classical electromagnetic simulations. For metallic nanoparticles, we are incorporating quantum transport models into the first-principles electromagnetic simulations to ensure accurate characterization of these particles. Based on both experimental and numerical investigations, we will identify candidate micro/nanoparticles that optimize the microwave contrast of malignant tumors.

Our third aim is to conduct experimental investigations of the microwave scattering and absorption properties of the micro/nanoparticle contrast agents in tissue-mimicking phantoms. We are currently optimizing our experimental synthesis techniques to synthesize a variety of nanoparticles and to modify their surface properties for desired dispersion properties. We plan to incorporate both standard and our in-house fabricated micro/nanoparticle contrast agents into synthesized “tumors” imbedded in simple breast phantoms and to characterize their enhanced scattering and absorption properties using monostatic radar techniques, fiberoptic temperature probes, and ultrasound transducers that externally measure the thermoacoustic response. In preliminary work we have experimentally characterized the thermoacoustic response of simple dielectric targets with various concentrations of microbubbles.

The results of this feasibility study will advance our understanding of microwave interactions with nanoparticle contrast agents, provide indisputable evidence of efficacy, permit subsequent development of enhanced microwave breast imaging technologies suitable for clinical trials, and ultimately lead to the establishment of low-cost microwave technologies as clinically relevant tools for breast cancer detection.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0629.

P11-9: NOTCH AS A DIAGNOSTIC TARGET FOR BREAST CANCER

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Background: The Notch signaling pathway represents a general mechanism of defining cell fate leading to cellular specialization and has been linked to aberrant growth of a variety of tumor types, including breast cancer.

Objective/Hypothesis: We hypothesize that Notch and Jagged-1 expression on breast cancer cells, as well as Delta-4 on breast tumor vessels present a diagnostic and therapeutic target for detection and treatment of breast cancer. Our combined objective is to utilize mouse models to establish whether (1) breast cancer cells and tumor vessels that express Jagged-1 or Delta-4 can be detected using a novel diagnostic platform and (2) tumor-derived proteins that shed upon Notch activation can be detected in serum of tumor bearing animals.

Specific Aims: Diagnostic Imaging of Jagged-1/Delta-4 Expressing Breast Tumors. Utilize a nanoparticle-based imaging platform (Diacovo) to detect for the elevated expression of the Notch ligands (Delta-like 4) in whole animals bearing murine or xenografted human breast tumors (Kitajewski). We have currently generated and produced soluble variants of Dll4 and Jagged1 for use in antibody preparation and for use in tumor modeling.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0362.

P11-10: GENETICALLY ENGINEERED BIONANOCONJUGATES FOR TREATING BREAST CANCERS

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Background and Objectives: The objectives of this work are (1) to integrate phage display technology and nanotechnology to develop bionanoconjugates that can selectively bind to breast cancer cells and be internalized into the cells; and (2) to evaluate the use of the bionanoconjugates in selective breast cancer cell destruction. The bionanoconjugates are assembled from phage proteins that are genetically engineered to recognize breast cancer cells and gold nanorods (AuNRs) that will kill the cancer cells upon exposure to near-infrared (NIR) light. Our work is based on the following observations. First, a NIR light absorbed by AuNRs can be converted into heat. Second, the NIR light has a tissue-penetration depth of several centimeters. Third, phage display can be used to identify peptides that can specifically target a desired cell.

Methodologies: We first use a phage-displayed random peptide library to perform biopanning in order to identify a peptide that can bind to and be internalized by SKBR-3 breast cancer cells. We then genetically fuse the cell-specific peptide to the phage. The fusion proteins are then immobilized on AuNRs to form bionanoconjugates. We will finally test the application of bionanoconjugates in cancer cell destruction.

Results to Date: Our results to date indicate that we could successfully identify the binding peptides and assemble them on AuNRs. Currently we are evaluating the stability of the bionanoconjugates and their application in cancer cell destruction. (C.1) Identification of SKBR-3 cancer cell-binding peptide: Based on the biopanning and DNA sequencing results, we found that phage displaying DGSIPWST and VSSTQDFP can bind to and be internalized into SKBR-3 cancer cell with greater affinity. We also observed a general amino acid pattern, aliphatic-hydroxyl-hydroxyl-hydroxyl-x-x-x-hydrophobic, in the binding peptides obtained. (C.2) Fusion and isolation of cancer cell-binding peptides: We fused the peptides to the phage and isolated fusion proteins. The successful isolation of the fusion protein was confirmed by gel electrophoresis and mass spectrometry. (C.3) Assembly of the cancer-cell-binding proteins on AuNRs: The as-prepared AuNRs are positively charged because cetyltrimethylammonium bromide (CTAB) forms a tightly packed bilayer on the surface. We applied layer-by-layer (LBL) self-assembly to assemble the cell-binding protein on AuNRs. We first adsorbed an anionic polymer (poly(styrenesulfonic acid sodium salt), PSS) on the CTAB-capped AuNRs, followed by adsorbing the protein on the surface. The resultant PSS-CTAB-AuNRs complex was water-soluble. The complex shows strong absorption peak at 701 nm, which originates from the AuNRs. The incubation of the complex with the protein resulted in an obvious spectral change in that the peak at 701 nm was red-shifted to 714 nm, suggesting that the cell-binding protein was successfully immobilized onto PSS-CTAB-AuNRs. Furthermore, a new peak appeared at about 280 nm, which is ascribed to the characteristic peak of protein.

Conclusions: Breast cancer cell binding peptides have been identified and immobilized on the surface of AuNRs to form bionanoconjugates. The bionanoconjugates will improve the ability to detect the cancer cells more efficiently than the conventional vectors and can selectively thermally heat cancer cells upon NIR irradiations, thereby providing a new method of treating breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0572.

P11-11: SINGLE-WALLED CARBON NANOTUBES TARGETED TO THE TUMOR VASCULATURE FOR BREAST CANCER TREATMENT

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Background and Objectives: This project explores a novel treatment of breast cancer that uses single-walled carbon nanotubes (SWNTs) in photodynamic therapy (PDT). SWNTs are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have very low levels of absorption of NIR light. To have a simple means of delivery of SWNTs to the tumor, it is proposed to target the SWNTs by conjugation with human annexin V. SWNTs that absorb NIR light strongly at 980 nm will be used, which penetrate more deeply in tissue than NIR light at lower wavelengths. Human annexin V is a protein that binds with high affinity to phosphatidylserine (PS) in phospholipid bilayers. PS is the most abundant anionic phospholipid of the plasma membrane and is tightly segregated to the internal side of the plasma membrane in most cell types. Recently, it has been found that PS is expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the outside surface of the vascular endothelium in normal organs. Thus, annexin V can be used to specifically target the endothelial cells of the tumor vasculature. The objectives of this project are to (1) produce by recombinant DNA technology human annexin V and conjugate the annexin V to SWNTs, (2) verify that the SWNT-annexin V complex will bind specifically to surface-immobilized PS *in vitro* and to the surface of human endothelial cells *in vitro* in which PS has been induced to be on the cell surface, and (3) demonstrate that endothelial cells with SWNT-annexin V bound can be killed by NIR light.

Methods: Annexin V will be produced by recombinant *Escherichia coli* and then purified to homogeneity. SWNTs will be dispersed in the presence of carboxymethyl-cellulose (CMC) to give a SWNT-CMC complex. Annexin V will be covalently coupled to CMC on the SWNTs using 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC). The binding of SWNT-annexin V to PS will be measured using PS immobilized on plastic microtiter plates and also using human endothelial cells *in vitro* in which PS has been induced to be on the cell surface by the addition of a low concentration of hydrogen peroxide. The cells with SWNT-annexin V bound will be exposed to NIR light at 980 nm for various times and power levels. At the end of each period of exposure to NIR light, cell viability will be determined.

Results to Date: Annexin V has been produced in *E. coli* and purified to homogeneity. The amino-terminal sequence (first six amino acids) of the protein has been found to be correct. Annexin V has been covalently coupled to SWNT-CMC using EDC, and the SWNT-CMC-annexin V complex has been found to stay stably suspended. Human endothelial cells have been obtained from the ATCC and successfully grown in the laboratory; aliquots of cells have been frozen for later experiments.

Conclusions: The project has the potential for a revolutionary impact on the treatment of breast cancer. There would be significantly fewer side effects than for conventional PDT, because the light-sensitive agent (SWNTs) is targeted specifically to the tumor. This therapy would be much less invasive than surgery and could replace surgery in some cases.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0563.

P11-12: PHOTO-DYNAMIC THERAPEUTICS BASED ON CN_x MULTI-WALLED NANOTUBES

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We demonstrate that nitrogen doped, multi-walled carbon nanotubes (CN_x-MWNT) prepared by chemical vapor deposition result in photo-ablative destruction of several cancer cell lines when excited by near infrared (NIR) irradiation. Further, we show that effective heat transduction and cellular cytotoxicity depends on nanotube length where effective NIR coupling occurs at nanotube lengths that exceed one-half the wavelength of the stimulating radiation, as predicted in classical antenna theory. We also demonstrate that this radiation heats the nanotubes through induction processes, resulting in significant heat transfer to surrounding media and cell killing at extraordinarily small radiation doses. This cell death was attributed directly to hyperthermia generated within the culture, since neither the infrared irradiation itself nor the CN_x-MWNT were not toxic to the cells. Finally we have applied this method directly to mouse models using xenografted tumors of several cell lines. We demonstrate resolution of tumors in our model with only one IR treatment and have examined final disposition and clearance of the nanomaterials within the animals using histological analysis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0681.

P11-13: PEPTIDE TARGETING OF NANOPARTICLES TO HUMAN BREAST CANCER CELLS IN VITRO AND IN VIVO

Deborah W. Knapp, Emily M. Haglund, Mary-Margaret Seale-Goldsmith, Deepika Dhawan, Jane C. Stewart, Christy L. Cooper, Lisa M. Reece, Donald E. Bergstrom, José A. Ramos-Vara, and James F. Leary
Purdue University

Background and Objectives: Nanomedical approaches are expected to provide earlier detection, more accurate staging, and improved therapy for breast cancer. Appropriately designed nanomedical approaches offer the advantage of selectively targeting breast cancer cells while avoiding damage to normal cells. The objective of this work was to develop nanoparticles that would selectively bind to and be taken up by breast cancer cells (BrCaNPs). The BrCaNPs are expected to serve as the base particle for the development of nanomedical detection and treatment tools for breast cancer. Two approaches were taken to target BrCaNPs to breast cancer: (1) an EphA2 antibody and (2) a breast cancer specific peptide. Both approaches were successful. Proof-of-concept studies involving the targeting peptide attached to quantum dot nanoparticles are summarized here.

Methods: Briefly, a breast cancer specific peptide (LTVSPWY) was conjugated to Qdot® 585 ITK™ amino (PEG) quantum dots (Invitrogen, approx. diameter 15 nm) by utilizing the NH₂ group using standard peptide coupling conditions to produce the BrCaNPs. The uptake of the BrCaNPs by human breast cancer cells (SKBR-3 and MCF-7) was studied. MCF-7 cells lack the target sequence and were included as a negative control. Other controls included: Qtracker system positive control (Invitrogen) and Qdots without targeting peptide as a negative control. *In vivo* studies, to determine the distribution and toxicity of BrCaNPs, were performed in the Purdue Cancer Center Drug Testing Facility. SKBR-3 cells were implanted SQ into 4-5 wk old, male, nu/nu athymic mice. Following the development of palpable tumors, BrCaNPs (100 µL, 200 nM solution, dose selected from pilot study) were injected via tail vein into the mice bearing SKBR-3 xenografts (or control xenografts). Mice (3 mice/group) were euthanized at 15 min, 1 hr, 3 hrs, 1 day, and 7 days. Mice were observed daily for toxicity, and necropsy with histopathology was performed at the time of death. BrCaNP distribution was determined (fluorescence and confocal microscopy) in tumor and normal tissues and in body fluids.

Results to Date: In the *in vitro* studies, the BrCaNPs were found in all SKBR-3 cells (cell membrane and cytoplasm) with negligible uptake in negative control MCF-7 cells. Similarly, the BrCaNPs were identified in cancer cells in SKBR-3 xenografts in athymic mice. No toxicity to the mice was detected.

Conclusions: In conclusion, these proof-of-concept studies demonstrate the ability to design BrCaNPs that will selectively target breast cancer cells. This sets the stage for the design and development of BrCaNPs on similar (yet biodegradable) core particles that will include detection and treatment components, and the targeting component.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0644; Purdue Cancer Center; and National Aeronautics and Space Administration.

P11-14: CURCUMIN-NANOGELS AND CURCUMIN-CARBON NANOTUBES AS MORE EFFICIENT FORMULATION OF CURCUMIN, A POPULAR ANTICANCER DIETARY SPICE

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Targeted drug delivery is a promising strategy to improve both the efficacy and safety of treatment. This is especially attractive for cancer therapy as most anti-cancer drugs have only marginal therapeutic index. In addition, the problem of efficiently delivering drugs, especially those that are hydrophobic or water-repellant, to tumors has long challenged scientists to develop innovative delivery systems that would keep these drugs intact until reaching their targets. Our work centers on one of the natural compounds, the potent anticancer agent curcumin that specifically kills cancer cells, while being non-toxic to the normal cells. Curcumin is one of the most well-known naturally occurring compounds commonly called turmeric. Since curcumin is a natural hydrophobic polyphenol its bioavailability has been a challenge for its therapeutic efficacy. The focus of our research was to develop a novel curcumin-encapsulated nanodevice aiming to increase the drug's solubility and cancer cell cytotoxicity. Two chemically different inorganic nanomaterials, single-walled carbon nanotubes (SWCNT) and colloidal nanogels (NG), were employed for this aim. A specific formulation of the polymers in nanogels was adjusted to encapsulate the anticancer natur-

al agent curcumin. Nonencapsulated compound was removed by dialysis. Curcumin-nanogel (C-NG) complexes were size fractionated. The particles with the size between 100–200 nm were used for further modifications with Rhodamine.

In addition, SWCNT solution was prepared, characterized by transmission electron microscopy and loaded with curcumin. Using Amnis imaging technology we have demonstrated the intracellular localization of the C-NG compared with the curcumin alone. In vitro experiments indicated that whereas both nanocarriers themselves are non-toxic to the breast cancer cells, the loaded nanoconstructs in particular, in nanogel formulation (NG-C), are significantly more cytotoxic than curcumin alone. NG-C was 70%–85% more cytotoxic to the cells at even lower than IC50 concentration than curcumin alone. This was confirmed morphologically by acridine orange/ethidium bromide staining. We conclude that our nanoformulation of anticancer natural agent curcumin offers an innovative, cost-effective nanodevice that is non-toxic to the normal cells and can be used in much higher doses than conventional chemotherapeutic drugs. Hence, we believe that our proposed strategy represents a promising approach for the treatment of breast cancer with the potential of a greater success than currently used therapy. In future work we are planning to functionalize the loaded nanocarriers with antibodies specific for surface receptors of breast cancer cells such as IGFR and HER2 for more specific cancer cells targeting.

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P11-15: BREAST TUMOR LOCALIZATION OF MAGNETICALLY RESPONSIVE NANOPARTICLE DRUG CARRIERS

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Background: Adjuvant chemotherapy has clearly shown its value in breast cancer treatment both in improved patient survival and in conjunction with breast conservation surgery. Nevertheless, local recurrences, particularly skin recurrences, are still a significant life-threatening problem in some patients, inadequately addressed by combined radiotherapy to the margin and CTX. Since such chemotherapy usually entails systemic administration of nontargeted agents, we proposed to initially develop and evaluate a novel nanoparticle (NP)-based preclinical therapeutic strategy employing physical vectoring of chemotherapy, namely, administering a magnetically responsive NP (MNP) preloaded with paclitaxel (TXL) and application of an external magnetic field appropriately focused to the tumor.

Methodology: A tandem array of Nd/B/Fe permanent magnets (22 mm diameter cylindrical magnet in combination with a pyramid-shaped magnet, with a ~ 3 mm square peak), rated at ~5600 Gauss at the surface was used to enhance extravasation of intravenously-injected single-domain, magnetite-based MNPs (~15 nm diameter). Female nude mice were implanted in the mammary fatpad with MDA-MB-435 cells, and when these tumors reached ~7–10 mm diameter, the mice were entered into a localization study. In the main experiments, mice were injected intravenously, using tail vein catheters with 150 µL of 1000 µg/mL FITC-labeled MNPs followed by an equivalent volume of a phosphate-buffered saline flush. This MNP dose was previously determined to create magnetic resonance imaging (MRI) susceptibility artifacts in T2* MR images in our studies in orthotopic human ovarian carcinoma models. Mice were imaged before the experiment and either not subjected to magnetic influence during and after MNP injection or were positioned such that the evident tumor was juxtaposed next to the point of the pyramid component of the tandem magnet. The magnet was put into this position prior to FITC-MNP administration and throughout the subsequent hour following the end of the flush. Mice were again imaged after magnetic localization ceased or 1 hour post-MNP injection in the case of the nonmagnet controls.

Results: The results of the MRI studies compared images of a mouse that did not receive magnetic influence with those of a mouse that had the tandem magnet in place during and after MNP injection. On the basis of these comparisons, regions of interest (ROIs) were defined in the liver and tumor of the latter mouse. The ROIs in the post-injection images displayed MNP-induced susceptibility artifacts consistent with MNP localization in these ROIs.

Conclusions: We have demonstrated that MNPs can be magnetically vectored into the tumoral/peri-tumoral environment in an orthotopic human breast carcinoma/nude mouse model using conventional, commercially available rare earth permanent magnets. This is a necessary milestone on the pathway to using these MNPs as a drug delivery platform. We have also prepared (under subcontract) a lead formulation of MNP-TXL currently under evaluation.

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P11-16: THE SELF-GUIDED NANOCOMPOUNDS TARGETING BREAST CANCER CELLS

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Main Idea and Background: It is well established that the development of breast cancer tumors are accompanied with hypoxia and acidosis. The physiological difference between normal and tumor tissues provides an opportunity for development of novel diagnostic and therapeutic agents specifically targeting cancer cells. However, the acidic extracellular environment in breast tumors has not been properly explored yet probably due to a lack of compounds that dramatically change their properties in the range of pH 6.0–7.5. Recently we designed the pH Low Insertion Peptide (pHLIP), which acts as a nanosyringe, it inserts in cellular membrane and forms transmembrane helix at acidic extracellular pH (6.0–6.5) but not at normal pH. Our preliminary data demonstrated that the fluorescently labeled pHLIP was accumulated in breast cancer tumors established in mice. pHLIP can find cancer cells and insert itself in their membrane. No insertion occurs in normal cells (pH 7.4). The pHLIP can be used to deliver to or into cancer cells various compounds including diagnostic probes, drugs, radiation or photo-sensitizers and thermosensitizers. The later is particularly interesting since it allows to use near-infrared (NIR) light to heat selectively the cancer cells with attached thermosensitizers. The progress in nanotechnology leads to the development of new materials such as carbon nanotubes. Single wall carbon nanotubes (SWCNT) have very high absorbance in NIR region and may serve as nanoheaters.

Specific Aims: (1) To design and synthesize water soluble fluorescently labeled pHLIP-SWCNT, (2) to determine the biodistribution of pHLIP-SWCNT in mice with implanted breast cancer tumors, and (3) to demonstrate the therapeutic feasibility of use of pHLIP-SWCNT as NIR thermosensitizers.

Preliminary Results: At the first stage of our project, we developed the method of preparation of water-soluble pHLIP-SWCNT constructs and tested their heating ability in solution upon illumination with NIR laser. Currently we are studying a toxicity of synthesized compounds. In a short term, we did not observe any toxic effects on mice. We also did not find any toxic effects in cell culture of murine breast adenocarcinoma cells, SRL-2116 line, from American Type Culture Collection in absence of laser radiation. We expect that laser radiation would induce heating of carbon nanotubes tethered to the membrane by pHLIP and disruption of membrane, which could be detected by measuring a leakage of membrane. The experiments will be done on liposomes and live cells. The results will be analyzed and presented at the Conference.

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P11-17: DEVELOPMENT OF A NANOCRYSTAL-BASED DRUG DELIVERY SYSTEM

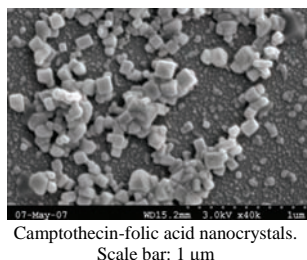
Tonglei Li, Christin P. Hollis, Hong Yang, and Kimberly W. Anderson
University of Kentucky

Poor solubility and severe side effects are two major roadblocks that limit the delivery and thereby the therapeutic efficacy of many anticancer drugs. In this project, we aim to deliver solid-based, nanosized, and multifunctional therapeutic and diagnostic systems to the tumor site. We hypothesize that because of the ligand-induced receptor-mediated endocytosis, hybrid nanocrystals can be taken up by cancer cells directly. Taking advantage of the enhanced permeability and retention (EPR) effect, we believe nanocrystals can accumulate around tumor sites, achieving the sustained release due to their deposit and being possibly recycled from dead cells. The main goal of this study is to test whether hybrid nanocrystals are more effective than current delivery systems (e.g., Taxol®) treating breast cancer in vitro and in vivo.

As such, we have developed crystal growth methods for producing nanosized drug particles. Growing nanosized organic crystals pose a great challenge, requiring significant research of proper growth conditions. Compared to inorganics, organic molecules have weak intermolecular interactions (mainly van der Waals forces) in the solid state. An organic crystal tends to dissolve to various concentrations in common solvents including water. Even for poorly soluble drugs, the solubility cannot be totally ignored. During the crystal growth in a liquid environment, because of the dynamic equilibrium between dissolving and precipitating, the larger crystals will grow even larger while the smaller crystals will become even smaller and eventually disappear. The key for developing nanosized organic crystals is not only to control the crystal size but also to reach a narrow distribution of crystal size. To produce a stable nanocrystal system from solution, the nucleation process has to be spontaneous so that all the nuclei have similar sizes and can continue to grow uniformly. Several crystallization methods have thereby been developed in the study to carefully control the nucleation step of crystallization. By testing various combinations of growth

conditions, we are able to obtain repeatable results of nanosized crystals that show acceptable stability behaviors for further testing. The figure shows camptothecin-folic acid nanocrystals produced by our antisolvent method.

We are continuing to improve the methods so that the stability of nanocrystals can last days and months. Concurrently, we are testing the nanocrystals in vitro regarding the cellular uptake and viability.



Camptothecin-folic acid nanocrystals.
Scale bar: 1 μ m

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0326.

P11-18: NANOPOROUS SILICON PARTICLES AS A MULTISTAGE DELIVERY SYSTEM FOR IMAGING AND THERAPY OF BREAST CANCER

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Background: Nanofabricated devices designed to carry drug and contrast agents to breast cancer cells are surface modified with targeting moieties that recognize unique or abundantly expressed molecules on the surface of tumor cells. However, major obstacles such as enzymatic degradation, uptake by professional phagocytes and the reticular endothelial system, and the vascular endothelium could hinder the ability of nanoparticles to reach the tumor site. To overcome these biological barriers, we have developed a multistage delivery system comprised of biocompatible porous silicon particles that encapsulate nanoparticles for protection and transport to tumor-associated vasculature. Degradation of the carrier silicon particles at the tumor site leads to the release of the nanoparticles, with subsequent extravasation to the tumor site through intravascular gaps and fenestrations. Herein we test the interaction of the carrier silicon particles with vascular endothelial cells to determine the effect of particle size and surface chemistry on cell interaction.

Methods: Based on rational design of particles for optimal margination and adherence to the vascular endothelium, we have fabricated hemispherical silicon particles in 2 sizes, 1.6 and 3.2 μ m. In addition to size, the effect of surface chemistry, as well as the presence of serum on binding of the particles to vascular endothelial cells was examined by FACS analysis, confocal, transmission, and scanning electron microscopy.

Results: The interaction of the silicon carrier particle with endothelial cells was altered by modifying the surface chemistry of the particle. Both oxidized (negatively charged) and apes-modified (positively charged) particles were taken up by human umbilical vein endothelial cells via phagocytosis. This uptake was inhibited by blocking actin formation with cytochalasin B. Modification of the particle surface with poly(ethylene glycol) inhibited both uptake and interaction of the particles with the cell surface. While both sizes of particles were internalized with similar kinetics, the presence of serum had a negative impact on binding and phagocytosis of oxidized particles. Aptes-modified particles were internalized by cells in presence and absence of serum.

Conclusions: Endothelial cells are an important component of the phagocytic system and avoidance of nonspecific uptake needs to be incorporated into the design of micron-sized carriers system. Modification of particle surface chemistry and the presence of serum affect interaction of particles with vascular endothelial cells. Particles can be designed to either arrest at the cell surface or undergo internalization for intracellular release of nanoparticles. Further modification with targeting ligands will address the specificity of delivery.

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P11-19: COMPOSITE NANOPARTICLES FOR TARGETED DELIVERY TO BREAST SOLID TUMORS

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Breast cancer is one of the leading causes of death in the world. One way of obtaining control over breast tumor growth is through selective killing of cancer cells with chemotherapeutics. These drugs should either (1) exhibit exquisite therapeutic activity and no side effects or (2) be delivered precisely to the tumor cells at increased concentrations and away from sites of dose-limiting toxicity. Numerous investigators are turning to nanotechnology for the development of multifunctional delivery sys-

tems capable of site-selective release of therapeutic compounds. While eventually nanoconstructs will, most certainly, have a bright future in medicine, current applications of nanoparticles in cancer therapies, however, are only slowly developing and are few in numbers. This is, in part, due to lack of research personnel trained at the interface of nanoparticle synthesis, characterization and preclinical cancer research.

The objective of this postdoctoral research is to demonstrate that large quantities of anticancer agents can be safely and selectively to estrogen receptor-negative (ER-) breast tumors using novel intelligent bi-particle nanotech drug delivery systems (nanoDDS). Proven cancer drugs like Adriamycin[®] are very effective at killing this type of cancer but are toxic to normal tissue; therefore, they can no longer be administered in large, therapeutically viable doses. Currently, the research is focused on the development of methods for: (1) incorporating drugs such as Adriamycin and Taxol[®] into the pores of iron oxide-silica composite nanoparticles (which can be traced by magnetic resonance imaging) and (2) controlling the drug release using elastic triblock copolymers. Inside the bloodstream (neutral pH) folded elastic polymers will block the particle pores, preventing drug release as if they were "trap doors" to the pores of the nanoparticles. Upon entering the acidic microenvironment of the tumor, these polymers will unfold, opening the pores and releasing the drugs. The element of novelty in this research comes from employing existing scientific principles and materials to create a novel nanotech drug delivery system. Therefore, the major challenge of this project is to integrate the main nanoDDS components: nanoparticles, biopolymers, and chemotherapeutics. The results to date include successful large-scale monomer synthesis, and the current work is focused on optimizing the polymerization conditions for the synthesis of elastic triblock copolymers.

The results of the proposed research will make a significant impact on both concepts and methods that drive the fields of breast cancer research and cancer nanotechnology. First and foremost, a working bi-particle nanoDDS will present a method for site-selective delivery of Adriamycin and Taxol to ER- breast tumors, which will warrant higher efficacy and reduced toxicity of these drugs, thereby contributing to the improvement of life for patients with breast cancer. Secondly, the ability to image the system and correlate tumor localization with efficacy can facilitate personalized therapy. The results of biological evaluation of polymer-inorganic nanoparticle moieties will provide the cancer research community with a standard for evaluation of similar systems. Furthermore, these studies can lead to the development of similar platforms for the delivery of a variety of chemotherapeutics for breast cancer therapy.

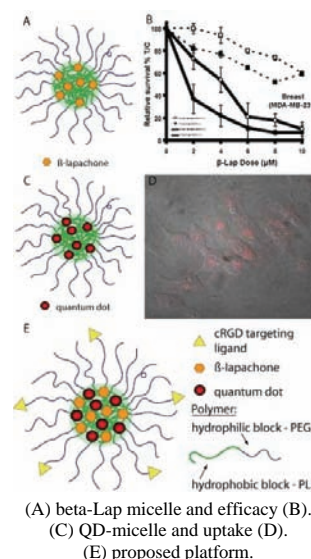
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0607.

P11-20: MULTIFUNCTIONAL BETA-LAPACHONE NANOTHERAPEUTICS FOR BREAST CANCER TREATMENT

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Presently, chemotherapy suffers from agent insolubility and systemic toxicity. Our goal is to target breast tumors: (1) pharmacokinetically through the use of polymer micelles to ensure drug stability and targeted delivery (via cRGD encoding) and (2) pharmacodynamically using a drug (β -lapachone) that is activated by an enzyme overexpressed in tumors (NQO1). We hypothesize that β -lapachone (β -lap) micelles will be an effective nanotherapeutic platform for breast tumor treatment and that quantum dot (QD) incorporation into micelles will allow for accurate localization of target tissues in vivo and effective evaluation of therapy.



Poly(ethylene glycol)-b-poly(D, L-lactic acid) (5kD-5kD) was synthesized using a ring-opening polymerization method, and a film sonication procedure was used to fabricate β -lap micelles. Size was determined using dynamic light scattering (DLS), β -lap encapsulation demonstrated via ¹H-NMR, and release studies performed in PBS at pH 7.4. β -lap micelle cytotoxicity was examined in NQO1(+/-) MDA-MB-231 breast cancer cells treated for 2 hr. QD-containing micelles were fabricated and their sizes characterized via DLS and TEM. Fluorescent yield was determined via fluorescence spectroscopy. In vitro uptake of QD-micelles was examined in SLK tumor cells incubated with micelles and imaged via confocal microscopy.

β -lap micelles possessed small size (~ 42 nm), core-shell morphology and diffusion-based release kinetics with a time for 50% of drug release ($t_{1/2}$) of 18 hr. After a 2 hr incubation with β -lap micelles, a marked increase in toxicity was shown in NQO1 overexpressing MDA-MB-231 cells over NQO1-null MDA-MB-231 cells. Compared to free β -lap, micelle-delivered β -lap showed decreased toxicity in both NQO1(+/-) MDA-MB-231 cells. QD micelles showed conservation of fluorescence intensity, as well as small size (~ 49 nm) and adequate encapsulation of QDs within micelles. In vitro uptake studies show tumor internalization of QD micelles after a 72 h exposure.

We report the development of β -lap micelles for treatment of breast tumors. Preliminary in vivo results show that β -lap micelles effectively maintained A549 lung xenograft tumors in mice (average volume of 116 ± 75 mm³ for 58 d). Moreover, β -lap micelles did not cause appreciable levels of hemolysis when compared to β -lap complexed with HP β -CD, a highly hemolytic solubilizer. Future studies will focus on micelle functionalization with QDs for treatment evaluation and tracking and with a cRGD ligand for active targeting to tumor sites.

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LIFESTYLE AND NUTRITION I

Poster Session P12

P12-1: MECHANISM OF BREAST TUMOR GROWTH INHIBITION BY ω -3 FATTY ACID DIET

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Omega (ω)-3 fatty acids (DHA and EPA) present in fish oil diet inhibited growth of MDA MB 231 human breast cancer cells in vitro. Mice maintained on a diet supplemented with 10% fish oil were resistant to tumor growth when inoculated with MDA MB 231 cells. There was a significant increase in the tumor suppressor protein PTEN in the tumors formed in these mice maintained on a fish oil diet. In addition, the fish oil diet inactivated the pro-survival PI 3 kinase and Akt kinase in the breast tumor tissues. The apoptotic signals were also activated in the tumor samples isolated from fish-oil-diet-fed mice. Using both western blotting and an enzymatic assay for detecting activated caspase 3 in tumor lysates, we detected increased caspase 3 activation in tumors of mice fed fish oil diet, confirming increased apoptosis in these tumor samples. A Western blot analysis using an antibody raised against activated (phosphorylated) form of the 65kD subunit of NF κ B showed significantly less activated p65 expression in fish oil tumor samples compared to the control tumor samples. Next DNA binding ability of activated NF κ B, which measures NF κ B transcriptional activity, was examined by Electrophoretic Mobility Shift Assay (EMSA) using nuclear extracts isolated from MDA MB 231 cells treated with DHA and EPA and a radioactive oligonucleotide probe containing NF κ B binding sequence. NF κ B DNA binding ability was significantly inhibited by DHA and EPA treatment of the breast cancer cells indicating that these fish oil ingredients are effective in reducing the DNA binding ability of NF κ B transcription factor essential for transactivation of several antiapoptotic genes in cancer cells. NF κ B induces transcription of antiapoptotic protein BclXL. To finally prove that DHA and EPA can inhibit NF κ B-mediated expression of antiapoptotic genes, we transfected MDA MB 231 cells with a BclXL promoter-driven luciferase plasmid in the presence or absence of an expression plasmid for p65 subunit of NF κ B. The transfected cells were treated with DHA or EPA or left untreated. BclXL gene expression was significantly induced by p65 subunit of NF κ B in untreated control cells. However, DHA and EPA treatment of these cells significantly reduced activation of BclXL gene expression by NF κ B. Taken together, we demonstrate for the first time that fish oil diet can intercept important proliferative signals in breast cancer cells and activate the apoptotic pathway to control the growth of breast cancer cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-04-1-0693; National Institutes of Health R01; and Veterans Affairs MERIT Review and Morrison Trust.

P12-2: ω 3 POLYUNSATURATED FATTY ACIDS (FISH OIL) ATTENUATE THE ESTROGEN-PROMOTED GROWTH OF BREAST CANCER CELLS BY MODIFYING THE ACTIONS OF A MEMBRANE-BOUND ESTROGEN RECEPTOR (GPR30)

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GPR30 is a member of the G protein-coupled receptor superfamily and mediates estrogen receptor-dependent kinase activation as well as transcriptional responses to that hormone. GPR30 also acts independently of the intracellular estrogen receptor to promote activation of the protein kinase A (PKA) pathway. This laboratory has demonstrated that Gs α , the G protein mediating the effects of GPR30, is sequestered in cholesterol-rich cytoskeletal-associated membrane domains (lipid rafts) and that this sequestration attenuates the action of Gs-coupled receptors like GPR30. The ω -3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are putative antioncogenic factors that inhibit the growth of human breast cancer cells. We hypothesize that the ω -3 fatty acids might exert their protective effect by increasing sequestration of Gs α in lipid rafts, limiting the actions of estrogen through GPR30. To explore the modulation of breast cancer cell growth by ω -3 fatty acids, we examined the effects of EPA and DHA on proliferation of the estrogen-responsive MCF-7 human breast cancer cell line. Three days treatment of MCF-7 cells with EPA or DHA inhibited cell proliferation relative to controls. The GPR30 agonist, G-1, could increase the proliferation of MCF-7 cells around 15% compared to control group after overnight treatment, and this was blocked by DHA treatment of the cells. These results give rise to a possibility that inhibition of the GPR30 signaling pathway may be a mechanism for the possible chemoprotective effects of ω -3 fatty acids in breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0670.

P12-3: ABSTRACT WITHDRAWN

P12-4: MECHANISMS OF BREAST CANCER ASSOCIATED WITH OBESITY

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Breast cancer incidence increases with age, but plateaus at a time that coincides with the onset of menopause. However, obesity is linked to a further linear increased risk of breast cancer incidence following menopause and is associated with a poorer subsequent survival for women who have breast cancer. One hypothesis suggests that circulating testosterone is converted to estrogen by aromatase in preadipocytes located in adipose tissues throughout the body. This would imply that obesity would result in higher localized estrogen thus leading to increased breast cancer incidence. This hypothesis, while attractive, remains to be rigorously proven. An alternative and equally plausible hypothesis is that the tumor-promoting effects of obesity may be occurring through nonestrogenic mechanisms. Recent research on adipocytes has demonstrated that these cells are potent endocrine cells that produce many hormones and factors that differ between the lean and obese states. In obese states, we have found a dramatic influx of macrophages in the adipose tissue, resulting in an inflammatory state that is thought to promote local and systemic insulin resistance that is also observed in human fat depots, but in mice we did not observe significant macrophage infiltration in inguinal fat pads. While in human obesity, macrophage infiltration has been reported to occur in subcutaneous fat. Since the influx of macrophages is thought to occur, in part to increased expression of monocyte-chemoattractant protein-1 (MCP-1) in obese adipose tissue, which is well known to recruit monocytes, we are currently interested in modeling this macrophage recruitment in mammary stroma to understand its role in obesity-related breast cancer development.

We have developed a procedure to grow normal human breast tissues in a mouse fat pad by first humanizing the mouse stroma with immortalized human reduction mammary fibroblasts (RMFs) and subsequently implanting a mixture of primary human breast epithelial cells (HMECs) and additional RMFs that have been resuspended in a collagen/ECM mixture. Following injection, HMECs develop into ductal and lobular structures that are present in the normal human breast but not in the normal mouse mammary gland. Using this model, we next investigated the role of the stroma in the developmental process of human breast tissue. Consequently, we generated several different human breast stromal fibroblast cell lines that overexpressed various growth factors that are known to have important effects on breast development and cancer pathogenesis. Using immortalized fibroblasts that overexpressed HGF or TGF β , we observed the outgrowth of unusual human mammary tissues, indeed growths that closely recapitulated the various presumed early stages of human breast cancer and even invasive carcinomas. In contrast, no lesions or tumors were observed in any instance in which normal primary human breast fibroblasts were admixed with these breast epithelial cells prior to engraftment into growth factor-releasing stroma. Instead, only normal ductal and lobular structures were observed. These findings revealed the important role of breast stromal fibroblasts in normal and malignant breast development. More recently, we have extended these findings with primary uncultured HMECs that have been genetically engineered to express specific oncogenes that are involved in breast cancer pathogenesis.

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P12-5: LINKING OBESITY TO BREAST CANCER METASTASIS: A LEPTIN-SHIP2-CXCR4 AXIS

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Background and Objectives: Obesity predisposes to the development of insulin resistance, type II diabetes and cardiovascular diseases. Epidemiological studies indicate that obese individuals are prone to increased risk for many types of primary cancer (1), nodal metastasis, recurrence, and poor prognosis of breast cancer (2). Molecular mechanisms underlying this risk are not yet clear; however, hormonal imbalance (insulin, estrogen, and leptin) could be a contributing factor (3,4). Signaling crosstalk between metabolic pathways and the oncogenic/metastatic pathways and deregulation of this crosstalk during obesity is thought to increase cancer risk. To understand the relationship between obesity and breast cancer metastasis, we investigated the role of a negative regulator of insulin signaling, SH2-containing 5'-inositol phosphatase (SHIP2), in leptin and CXCR4 signaling. SHIP2 is upregulated by high-fat diet, obesity, and diabetes. SHIP2 is overexpressed in breast cancer cells and regulates adhesion/cell spreading and receptor endocytosis. Leptin, an adipocytes-secreted hormone, increases SHIP2 protein levels in MCF-7 breast cancer cells. CXCR4, a G-protein coupled receptor, is also overexpressed in breast cancers, and its signaling promotes cancer cell survival, invasion, homing, and proliferation at sites of metastasis. Signaling from CXCR4 is downregulated through ligand-dependent endocytosis. Because of defects in general endocytosis in SHIP2 overexpressing cells, CXCR4 signaling is hypothesized to be potentially prolonged. Specific aims of this

project were to (1) determine the effect of leptin on SDF-1 α -CXCR4 signaling and examine the role of SHIP2 in mediating this effect and (2) investigate the contribution of SHIP2 in metastasis.

Methodology: RNA interference approach was used to silence the endogenous SHIP2 expression in metastatic MDA-231 breast cancer cells followed by in vitro and in vivo analyses as described next.

Results: In metastatic variants of MDA-231 cells, CXCR4 levels were induced by leptin and SDF treatment. Upon transient and stable SHIP2 RNAi, CXCR4 levels were reduced. Furthermore, decreased SHIP2 caused reduced cell proliferation, clonogenicity, and cell migration. SHIP2 silencing also reduced tumor growth and spontaneous lung metastasis in vivo. Thus, SHIP2 appears to be central to a molecular network that links high-fat diet/obesity-breast cancer metastasis.

Relevance: SHIP2 is a unique drug target with dual application whose inhibition could limit metastasis and restore normal metabolic signaling during obesity. CXCR4 is an important determinant of metastasis whose putative regulation by SHIP2 in cancer cells is a novel and rational theory that links metabolic signaling to metastatic signaling. Our data support a molecular axis between leptin, SHIP2 and CXCR4 thereby mechanistically linking increased metastasis and poor survival rates with high-fat diet and obesity. These findings will form the basis of new efforts to develop pharmacological inhibitors of SHIP2.

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P12-6: FOODS AND FOOD GROUPS ASSOCIATED WITH FOLLICULAR TOTAL AND FREE ESTRADIOL LEVELS IN PREMENOPAUSAL WOMEN

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Background and Objectives: We found a significant positive relation between intake of animal, but not vegetable, fat, and risk of breast cancer (p-trend = 0.002) among Nurses' Health Study II (NHS II) participants. Further, we observed a significant positive association between red meat intake and risk of estrogen/progesterone receptor positive but not estrogen/progesterone receptor negative premenopausal breast cancer (p-trend=0.001). In addition, premenopausal women in the top (versus bottom) 25% of follicular total (T) and free (F) estradiol (E2) levels had a significantly increased risk of breast cancer [RR=2.1, 95% CI=(1.1–4.1), p-trend=0.08; RR=2.2, 95% CI=(1.2–4.2), p-trend=0.03, respectively]. Therefore, in this study we aimed to identify foods and food groups associated with increased levels of follicular TE2 and FE2 levels in 592 premenopausal women from the NHS II. Participants comprised controls from a breast cancer nested case-control study and women who were included in a reproducibility study. In particular, we hypothesized a positive association between red meat intake and follicular TE2 and FE2 levels.

Brief Description and Methodologies: Servings of foods and food groups consumed per day were calculated using the average of two semi-quantitative food frequency questionnaires completed in 1995 and 1999 (both with more than 130 food items) to best represent long-term intakes. Using partial Spearman correlations, we identified foods, food groups, breast cancer risk factors, and potential confounders that were associated with our hormones of interest ($r>0.1$) for entry into regression analyses. Models predicting follicular sex hormone were adjusted for blood collection factors, total caloric intake, age, BMI, weight change since age 18, ovulatory status, duration of oral contraceptive use, and smoking status.

Results to Date: Women had mean (\pm sd) age of 43.5 (\pm 3.9) years; BMI of 25.1 (\pm 5.3) kg/m²; height of 143.1 (\pm 5.8) cm; and total caloric intake of 1,834 (\pm 484) kcal. We did not find any association of red meat intake with TE2 or FE2. We found significant positive associations between FE2 and intakes of high-sugar foods such as sweet rolls, coffee cake, pastries (p=0.03), and juices (other than apple, apple cider, orange and grapefruit juices) (p=0.03). In addition, intakes of crackers, Triscuits, and wheat thins were inversely associated with TE2 (p=0.04). No other specific foods or food groups were associated with follicular E2 levels.

Conclusions, Including the Potential Impact on Breast Cancer Research and/or Treatment: We did not observe any association between follicular estrogen levels and red meat intake as hypothesized. However, our findings provide suggestions of adverse impact of certain types of foods on estrogen levels, also observed previously in other studies; in particular, high-sugar/refined-carbohydrate/low-fiber foods (i.e.,

breakfast pastries and some fruit juices). If these findings are replicated in other studies, they could serve as the basis for a randomized well-controlled dietary intervention study in premenopausal women to reduce follicular TE2 and FE2 levels. Studies such as this are important in developing dietary guidelines to reduce the risk of breast cancer in premenopausal women, given dietary intervention studies with premenopausal breast cancer as the endpoint are unlikely to be feasible.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9410 and National Cancer Institute.

P12-7: THE ω -6 AND ω -3 POLYUNSATURATED FATTY ACIDS AND MODIFIABLE BREAST CANCER RISK FACTORS

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Background/Objectives: Experimental and epidemiological evidence suggests that omega-6 (ω -6) polyunsaturated fatty acids (PUFAs) promote breast tumorigenesis whereas omega-3 (ω -3) PUFAs inhibit mammary tumor growth. Laboratory studies indicate that these two families of fatty acids influence breast cancer risk by impacting eicosanoid synthesis. In particular, when ω -3 PUFAs displace ω -6 PUFAs, prostaglandin E2 (PGE2) production is reduced, resulting in decreased aromatase activity and suppression of estrogen synthesis. Whether this effect on estrogen production can be observed in the circulation or in breast tissue, as reflected on a mammogram, is unknown. Therefore, using erythrocyte fatty acids as a marker of recent dietary intake, we sought to establish the relationship between the ω -6 and ω -3 fatty acids with serum estradiol and percent breast density, two well-established breast cancer risk factors. We hypothesized that ω -6 PUFAs are positively related and ω -3 PUFAs negatively related to both risk factors. Nonsteroidal anti-inflammatory drugs (NSAIDs) also inhibit PGE2 formation, therefore we further hypothesized that estradiol levels would be lower among NSAID users. NSAID data was not available at the time of mammogram; hence the relationship between NSAID use and breast density could not accurately be assessed.

Methods: To test our hypotheses we conducted a cross-sectional investigation of 260 participants enrolled in the Mammograms and Masses Study, a case control study on the determinants of breast density. Women were eligible for this ancillary study if they were breast cancer-free, postmenopausal, and not taking hormone therapy. Erythrocyte fatty acids were measured by gas chromatography. Serum estradiol was measured by radioimmunoassay, and breast density was estimated by manual planimetry. Analysis of covariance was used to test each hypothesis. Estradiol values were logarithmically transformed and a square root transformation was applied to breast density data to obtain normal frequency distributions. Geometric means were calculated by taking the anti-log of the least squares means after adjustment.

Results: In multi-variable adjusted analyses, we observed significantly lower levels of serum estradiol among current users of NSAIDs as compared to non-users of NSAIDs (user=17.7 pmol/L versus nonuser=21.2 pmol/L; p=0.03). Further, among non-users of NSAIDs, estradiol levels decreased with increasing tertile of erythrocyte total ω -3 PUFAs (lower tertile=24.3 pmol/L versus upper tertile=18.4 pmol/L; p trend<0.05) and rose with increasing tertile of total ω -6 PUFAs (lower tertile=16.0 pmol/L versus upper tertile=21.8 pmol/L; p trend=0.02). A marginally significant positive association was also noted between the ratio of ω -6 PUFAs to ω -3 PUFAs and serum estradiol (lower tertile=17.6 pmol/L versus upper tertile=22.9 pmol/L; p trend=0.06). However, the significant associations between fatty acid measures and estradiol were not noted among NSAID users. No statistically significant relationships were observed between any one of the ω -6 or ω -3 fatty acid measures and breast density.

Conclusions: In summary, reducing ω -6 PUFA intake, increasing ω -3 PUFA intake, or taking an NSAID may result in reduced estradiol synthesis and ultimately breast cancer risk. If confirmed, these findings could lead to the creation of chemopreventive guidelines and ultimately prevent the development of estrogen-dependent breast cancer.

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P12-8: OBESITY ACCELERATES MOUSE MAMMARY TUMOR GROWTH IN THE ABSENCE OF OVARIAN HORMONES

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Introduction: Obesity increases incidence and mortality of breast cancer in postmenopausal women. Mechanisms underlying this association are poorly understood. Suitable animal models are needed to elucidate potential mechanisms for this association.

Methods: To determine the effects of obesity on mammary tumor growth, nonovariectomized and ovariectomized C57BL/6 mice of various body weights (lean, overweight, and obese) were implanted subcutaneously with mammary tumor cells from syngeneic Wnt-1 transgenic mice. The lean, overweight, and obese mouse phenotypes had comparable percent body fat values to women that are considered lean (BMI <25), overweight (BMI 25–30), and obese (BMI >30).

Results: In mice, the lean phenotype was associated with reduced Wnt-1 tumor growth regardless of ovarian hormone status. Ovariectomy delayed Wnt-1 tumor growth consistent with the known hormone responsiveness of these tumors. However, obesity accelerated tumor growth in ovariectomized but not in nonovariectomized, animals. The obese phenotype was associated with higher levels of insulin, insulin-like growth factor (IGF) 1, and leptin while the lean mice, which had the lowest tumor growth rate, displayed significantly lower levels of insulin, IGF-1, and leptin.

Conclusions: Diet-induced obesity in a syngeneic mouse model of breast cancer enhanced tumor growth and increased circulating levels of insulin, IGF-1, and leptin specifically in the absence of ovarian hormones. These results support epidemiological evidence that obesity is associated with increased breast cancer incidence and mortality in post- but not premenopausal women. In contrast, maintaining a lean body weight phenotype was associated with reduced Wnt-1 tumor growth and decreased serum levels of insulin, IGF-1, and leptin regardless of ovarian hormone status. We propose that using a breast cancer mouse model of dietary modulation will allow further elucidation of the mechanisms by which obesity and hormonal status affect breast cancer incidence and progression.

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P12-9: BREAST CANCER RISK REDUCTION: EFFECT OF DIETARY FAT AND FATTY ACIDS ON PLASMA ESTROGEN AND TESTOSTERONE INDICES IN POSTMENOPAUSAL WOMEN

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Breast cancer, a sex hormone mediated cancer, presents a significant problem in the United States. It is important to develop safe and effective preventative strategies for this disease. Epidemiological evidence and animal studies show that dietary fat is associated with risk of development of sex hormone mediated cancer. Specifically that a high intake of omega-6 fatty acids increases risk while omega-3 fatty acids are associated with risk reduction. Although the associations between dietary fat and sex hormone mediated cancers is unclear, it is likely due to mechanisms of endocrine balance.

The primary objective of this investigation was to determine whether diets of varied fat and fatty acid content would favorably affect sex hormone distribution in postmenopausal women in a direction associated with reduced risk of sex hormone-mediated cancer development. The specific aims of this study are to evaluate the effects of total fat and omega-3 fatty acid intake on androgen and estrogen indices.

In order to evaluate these relationships we are conducting a well-controlled feeding study to evaluate dietary fat and fatty acid effects. The diets being tested in 8-week feeding periods include a "high risk" American diet (40% fat; HF), a low fat diet (20% fat; LF) and a low fat diet with supplemental omega-3 fatty acids (23% fat; ω 3). End-point measures of plasma sex hormones were obtained at baseline (BL), 4, and 8 weeks of each dietary treatment. Plasma estradiol (E_2), testosterone (T) and sex hormone binding globulin (SHBG) were analyzed by radio-immunoassay for 10 participants. The Estrogen and Androgen Indices (EI, AI) were calculated as the ratio of E_2 :SHBG and T:SHBG, respectively.

A trend for decreased concentrations from baseline to 8 weeks was detected for E_2 with the LF and ω 3 diets. Consumption of the ω 3 increased SHBG concentrations significantly from baseline to 8 weeks ($p = 0.04$). The EI showed a trend for increase from baseline to 8 weeks in the HF compared to the ω 3 diet at week 8 ($p = .08$). The AI was significantly reduced following the HF compared to LF and ω 3 ($p = .006$ and $.002$, respectively).

Preliminary data shows that within subjects, 8 weeks consumption of a ω 3 diet significantly decreased plasma E_2 and increased SHBG concentrations in postmenopausal women. Compared to HF, consumption of the ω 3 and LF diets significantly decreased E_2 concentrations between groups at 8 weeks. The HF diet resulted in a decreased AI and increased EI. The LF and ω 3 diets are associated with reduced free estrogen levels which may reduce risk for breast cancer.

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P12-10: ABSTRACT WITHDRAWN

P12-11: ACTIVE AND PASSIVE TOBACCO SMOKE EXPOSURE INCREASES THE SOMATIC MUTATIONAL BURDEN BEGINNING IN THE WOMB

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The development of human cancer, including breast cancer, is a multistep process driven by somatic mutations and epigenetic events that mimic their effects. The frequencies of such events can be increased by occupational, environmental, and medical exposures, although relatively few such agents have been unambiguously identified. This is likely due to the facts that carcinogenesis occurs over decades, involves multiple exposures and multiple events, and that biodosimetry for most potential exposures is nonexistent. We have previously shown that the somatic mutational burden of cancer patients is significantly elevated over matched disease-free controls, and others have shown that the traditional measures of genotoxicity, chromosome aberration analysis, and micronucleus analysis are predictive of cancer incidence. Thus, we are apparently better at identifying people at increased risk of cancer due to exposure than we are carcinogenic exposures themselves. There are two widely applied assays for locus-specific human somatic mutation, at the hemizygous X-linked gene for the purine scavenger enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) and at the autosomal (heterozygous) determinant for the MN blood group, glycophorin A (GPA). Using these assays, we show that tobacco smoke exposure is associated with an increased somatic mutational burden in a large ($N = 482$) pooled study of healthy individuals. We also demonstrate the effects of active smoking, as well as of passive exposure, in a population of young mothers and in their newborn babies. Molecular analysis reveals that both active and passive exposure to tobacco smoke induces point mutations in the DNA of children in utero, as well as the products of illegitimate V(D)J recombination, which are responsible for many of the molecular events underlying childhood leukemia. At the autosomal GPA locus, an inductive effect of tobacco smoke exposure on chromosomal missegregation can also be observed. Tobacco smoke therefore represents a widespread if not ubiquitous exposure that contributes to carcinogenesis throughout the body, beginning in the womb. This approach of functional population monitoring of potentially carcinogenic effects represents a more feasible and actionable tactic for the public health translation of genotoxicity assays than ongoing attempts to demonstrate an association of human cancer with individual agents, especially chemicals.

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P12-12: OBESITY, ENERGY BALANCE, AND BREAST CANCER: DIFFERENTIAL EFFECTS OF CALORIE RESTRICTION AND TREADMILL EXERCISE IN p53-DEFICIENT MICE

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Background: The prevalence of obesity, an established risk factor for postmenopausal breast cancer and many other cancers, has risen steadily for the past several decades in the U.S. Particularly alarming are the increasing rates of obesity among children, portending continuing increases in the rates of obesity and obesity-related cancers for many years to come. Unfortunately, the impact and underlying mechanisms of weight reduction/control via exercise or reduced calorie diet regimens on breast cancer development in obese women has not been well studied.

Objective: To fill this knowledge gap and facilitate the development of new breast cancer prevention strategies, we determined the impact (and underlying mechanisms) of obesity and its reversal by treadmill exercise (20 meters/min for 30 mins/day) or calorie restriction (CR; 70% of the energy intake of the controls) on spontaneous mammary tumor development in p53-wild-type (p53+/+) and heterozygous p53-deficient (p53+/-) Wnt-1 transgenic (TG) mice.

Results: Weight reduction/maintenance via CR significantly delayed tumor development in Wnt-1 transgenic mice regardless of p53 tumor suppressor status. In contrast, we found that treadmill exercise, which suppresses tumor development in multiple p53+/+ mammary tumor models, reduced adiposity but accelerated mammary tumor development in p53-deficient Wnt-1 TG mice. Thus, two energy balance modulating interventions (CR and treadmill exercise) exerted differential effects on mammary tumor development in the p53-deficient state. In a separate group of obese p53+/+ and p53+/- mice, we found that both interventions decreased adiposity (including decreased adipokines such as leptin) and improved insulin sensitivity, but only CR (and not exercise) reduced circulating insulin-like growth factor (IGF)-1 levels. Microarray analysis of mammary gland and abdominal adipose tissue from these mice revealed very different gene expression profiles when comparing CR and treadmill exercise in both p53+/+ and p53+/- mice. Ongoing analyses of these data suggest that the p53-dependent differences in mammary tumor response to CR and exercise may be linked to differences in IGF-1, hypoxia, and glycolytic pathways, and we are currently evaluating the interactions between these pathways.

Conclusion: These findings suggest, for the first time, the possibility of an important interaction between p53 gene dosage and physical activity that may influence the breast cancer preventive effects of exercise. In addition, the results suggest the effects of increased energy expenditure (i.e., exercise) does not have the same effects as decreased energy intake (i.e., CR) on mammary tumor development, hormones or gene expression profiles, particularly in the context of p53-deficiency. These findings have important implications for the development of mechanism-based strategies for preventing breast cancer.

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P12-13: IN VIVO AND IN VITRO STUDIES RELATING LEPTIN AND OBESITY TO BREAST CANCER DEVELOPMENT

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Obesity is a risk factor for postmenopausal breast cancer, particularly estrogen receptor (ER) positive tumors. In addition, elevated body weight affects latency and/or incidence of chemically induced and spontaneous mammary tumors (MTs) in rodents. We propose that leptin, synthesized in adipose tissue in proportion to fat mass, provides a direct link between obesity and breast cancer. Evidence to support this hypothesis is presented.

Experiment 1: Transgenic MMTV-TGF- α mice that develop hormone-responsive MTs were crossbred with *Lep* (leptin-deficient) or *Lepr* (leptin receptor-deficient) obese mice. TGF- α lean and obese mice were monitored for MT development until 104 wks of age. Neither TGF- α -*LepobLepob* (0/59) (*BCRT* 77:205,2003) nor TGF- α -*LeprdbLeprdb* (0/42) (*Exp. Biol. Med.* 229:182, 2004) obese mice developed MTs, while lean mice had a MT incidence of 59% (44/74).

Experiment 2: MMTV-TGF- α mice with intact leptin axes were fed a moderately high-fat diet from 10 weeks of age. Obesity-prone, i.e., mice that gained excessive weight relative to low-fat fed mice had shortened MT latency compared to obesity-resistant mice that consumed the high-fat diet but remained in the weight range of

low-fat mice (*Int. J. Obesity* 28, 956, 2004). In a follow-up study the same protocol was used resulting in similar MT outcomes and serum leptin was elevated in obesity-prone (10.56 ng/mL) compared to obesity-resistant (3.6 ng/mL) and low-fat (1.58 ng/mL) mice. Fat pad weights were positively correlated with serum leptin level ($r=0.84$, $p<0.0001$) and leptin receptor OB-Rb protein was expressed in MTs from all groups (*Breast Cancer Res.* 9, in press, 2008).

Experiment 3: The diet-induced obesity protocol was used in MMTV-neu mice that develop ER- MTs. As predicted there was no effect of body weight, body fat, or serum leptin levels on MT development (*Nutr. Cancer* 50, 174, 2004).

Experiment 4: Direct comparison of ER+ versus ER- MT growth was made using MCF-7 and MDA-MB-231 human breast cancer cells inoculated into dietary-induced obese athymic mice. There was little effect of obesity on MT formation from ER-cells. ER+ MCF-7 cells formed small MTs making it difficult to evaluate results (*Cancer Lett* 253, 291, 2007).

Experiment 5: To further understand the impact of leptin in relationship to ER status five human breast cancer lines were used for in vitro studies. All lines expressed leptin receptors but exhibited variable response to leptin attributed to ER status (*Int. J. Oncol* 30, 1499, 2007).

Experiment 6: To make direct comparisons between ER+ and ER- MTs, MDA-MD-231 ER- cells were transfected with ER- α . Several ER- α sublines that exhibited increased proliferation in response to leptin were identified. These cells formed MTs in athymic mice and growth was accelerated with estradiol implants (*Br. J. Cancer* 98, in press, 2008). These cells will now be inoculated into mice to assess the effect of body weight and serum leptin levels on MT development.

In conclusion, epidemiological studies are inconclusive as to whether serum leptin is associated with breast cancer. This is not surprising given the limited scope of these studies. However, results from our in vivo and in vitro studies indicate that leptin impacts breast/mammary tumorigenesis when leptin receptor signaling is intact but is further modulated by tumor characteristics including ER and/or HER2 status in conjunction with circulating growth factors.

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EPIDEMIOLOGY I

Poster Session P13

P13-1: IMPACT OF INSTITUTIONAL- AND INDIVIDUAL-LEVEL DISCRIMINATION ON MEDICAL CARE AND QUALITY OF LIFE AMONG BREAST CANCER SURVIVORS

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Breast cancer stage at diagnosis, treatment, and possibly also quality of life varies substantially across racial/ethnic groups, but the reasons for these disparities are incompletely understood and appear to be only partly attributable to individual or census area-based socioeconomic measures. New directions in research are needed to gain a more complete understanding of the factors responsible for racial/ethnic disparities in breast cancer outcomes. Research on the impact of discrimination may help to identify the underlying factors that are responsible for the disparities; however, this research has not yet been done and represents a new and innovative direction in research on cancer disparities. The objective of this study is to measure the prevalence and impacts of discrimination at the institutional and individual level to identify the causal mechanisms contributing to disparities in breast cancer diagnosis, treatment, and quality of life. The study comprises two components: developmental and application. In the developmental component, the specific aim is to (1) develop a survey tool tailored toward cancer patients for assessing discrimination in health care settings. In the application component, the specific aims are to (2) quantify the prevalence of individual- and contextual-level discrimination across racial/ethnic groups and (3) assess the effects of individual- and contextual-level discrimination on disparities in (a) late-stage diagnosis, (b) cancer treatment (including breast conserving surgery and adjuvant radiation), and (c) quality of life. The developmental component uses qualitative research, including 6 race/ethnic-specific focus groups and 21 qualitative (one-on-one) interviews, to develop an instrument tailored for breast cancer patients. The instrument will be cognitive-tested in 21 patients. We will then conduct a pilot test, including a reliability test-retest, with 70 patients to optimize instrument reliability. In the application component, we will conduct a cross-sectional epidemiologic study with 1,139 patients using a multilevel approach by incorporating individual- and neighborhood-level information including: (1) previously collected geographic information systems (GIS) data about the social and built environment and (2) telephone interviews. Study participants will be randomly selected through the population-based Greater Bay Area Cancer Registry. This project, based on individual- and institutional-level data, is novel in that it will examine the effects of previously unexplored factors, that is, discrimination, residential segregation, and neighborhood social and built environment on medical care and outcome measures for breast cancer across several racial/ethnic groups. A unique aspect of the study is the focus on institutional discrimination as a primary cause of disparities in breast cancer diagnosis, treatment, and quality of life; interactions among multiple patient and neighborhood characteristics; and the hierarchical nature of the characteristics. Our study will allow us to identify combinations of patient and neighborhood factors characterizing subgroups and the pathways leading to late disease detection, inadequate care, or poor quality of life.

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P13-2: A NEW INSTRUMENT TO MEASURE SYMPTOM DISTRESS IN WOMEN WITH BREAST CANCER

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Treatment for breast cancer is constantly evolving with subsequent alteration in symptoms, symptom distress, and symptom trajectory. Current instruments used to measure symptom distress were developed in the 1970's and 1980's, and do not adequately measure symptoms and symptom distress experienced by women with breast cancer because of changes in treatment protocols causing a multitude of different side effects not experienced decades ago. Therefore, the aims of this study were to: (1) identify the full scope of symptoms and symptom distress in women with breast cancer; (2) develop a comprehensive instrument to measure symptoms and symptom distress.

This cross-sectional, correlational study was guided by the theoretical framework authored by the University of California, San Francisco, named the Symptom Management Conceptual Model, specifically the dimension of symptom experience because it focuses on perception, evaluation, and responses to symptoms.

Using the Breast Care Center of Western New York, 50 women (0 to 6 months post treatment); 50 women (7 to 12 months post treatment) were recruited. They were given a battery of six psychometrically sound instruments: Mishel Uncertainty in Illness Scale, Breast Cancer Prevention Trial Symptom Checklist, Cancer Rehabilitation Evaluation System, McCorkle Symptom Distress Scale, Symptom Checklist-90-Revised Scale, and Functional Assessment of Cancer Therapy-Breast measurement. Demographic and clinical information were obtained from the medical record.

Descriptive statistics were used to analyze demographic and clinical data; rank ordering was used to identify the most prominent/persistent symptoms causing symptom distress in these women. The mean age of the women was 50 years, 74% were mar-

ried; 92% were White. Clinically, 88% had a ductal carcinoma; 59% had a lumpectomy with a sentinel node biopsy. All received Cytosin and Adriamycin followed by Taxol.

Analyzing the 6 instruments using rank-ordering identified 5 constructs: physical symptoms, menopausal symptoms, cognitive impairment, body image changes, uncertainty. Incorporating the symptoms that ranked the highest served as the foundation in developing a new instrument to measure symptom distress in women with breast cancer; field notes and anecdotal comments not measured by the 6 instruments were also incorporated in the new instrument.

This study is valuable in that a new and more effective instrument was developed to measure symptom distress in breast cancer women that can be utilized by health care providers to identify symptoms and symptom distress earlier and accurately with the initiation of more appropriate interventions. The new instrument will provide women with an opportunity to voice symptoms and distress that perhaps they would not mention ordinarily during an office visit. It could also serve to validate some symptoms and symptom distress experienced, because if it appears on an instrument, others must also be experiencing these symptoms! The life-long consequences of this diagnosis must be recognized, addressed, and managed by health care providers so that women can go on to live their lives.

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P13-3: ONE-CARBON METABOLISM AND SURVIVAL AMONG A POPULATION-BASED STUDY OF WOMEN WITH BREAST CANCER

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Breast cancer is the second leading cause of cancer mortality among women. Breast cancer survival may be predicted from tumor characteristics and host and lifestyle factors. Given its important role in DNA methylation and synthesis, one-carbon metabolism may affect breast cancer mortality. We utilized a population-based cohort of 1,508 women with breast cancer to investigate possible associations of dietary intake of B vitamins prior to diagnosis as well as 9 polymorphisms of one-carbon metabolizing genes and subsequent survival. Women newly diagnosed with a first primary breast cancer in 1996–1997 were followed for vital status for an average of 5.6 years. Kaplan-Meier survival and multivariate Cox proportional hazard regression analyses were used to evaluate the association between dietary intake of B vitamins, genotypes, and all-cause as well as breast cancer-specific mortality. We found that higher dietary intake of vitamin B₁ and B₃ prior to diagnosis was associated with improved survival during the follow-up period (p for trend = 0.01 and 0.04, respectively). The *MTHFR* 677 T allele or *BHMT* 742 A allele were also associated with reduced all-cause mortality (hazards ratio and 95% confidence interval: 0.69 (0.49–0.98) and 0.70 (0.50–1.00), respectively). ER/PR status modified the association between the *MTHFR* C677T polymorphism and survival (p for interaction = 0.05). The survival associations with one-carbon polymorphisms did not differ with the use of chemotherapy although study power was limited for examining such effect modification. Our results indicate that one-carbon metabolism may be an important pathway that could be targeted to improve breast cancer survival.

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P13-4: EVIDENCE THAT MAMMARY EPITHELIUM (ME) OF WOMEN FROM HIGH BREAST CANCER (BC) INCIDENCE POPULATIONS IS SUBJECT TO CHRONIC OXIDATIVE STRESS (OXs)

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The aim of this study was to test the notion that chronic OxS underlies our high breast cancer (BC) incidence and, more specifically, that there is a correlation of markers of chronic OxS ME and BC risk.

Rationale: Epidemiological evidence identifies definitively western industrialized lifestyle/environment as the most potent risk factor for sporadic BC. Many environmental factors implicated in carcinogenesis can contribute to chronic OxS, that is, to a state in which generation of reactive electrophiles exceeds cells' ability to be protected from oxidative DNA damage and from disruption by electrophiles of signaling pathways regulating cellular homeostasis. Such a state in the ME can be predicted to lead to mutations and aberrant expression of genes controlling cell replication, differentia-

tion, and death and to facilitate emergence of a malignant geno/phenotype. This model predicts a correlation in a population between prevalence of markers of chronic OXS in ME and BC incidence.

Study Design: Breast tissue from women without BC obtained at (1) forensic autopsy in the 1970s from 3 populations from the southwestern United States that differed significantly in BC incidence and (2) surgery for macromastia from a population with a high BC incidence typical for the United States. The electrophilic product of lipid peroxidation, 4-hydroxy-2-nonenal (4HNE), was used as an immunocytochemical marker of chronic OXS.

Results: Breast tissue sections from 247 donors were immunostained for 4HNE (69 American Indian and 82 caucasian women from the southwestern United States with a 3.5-fold difference in BC incidence and 96 women from a population with high BC incidence typical for the United States). An examination of tissue section by the conventional method of pathologists assigning semi-quantitative scores to their observations, confirmed our original observation of a high prevalence of 4HNE adducts in ME in high BC risk populations and even in some as young as 14. However, the method proved inadequate for quantifying differences in the prevalence and intensity of 4HNE staining among and within the different populations. Hence, we directed our efforts to set up 2 recently developed cytometric methods. With one of these, we can now obtain histograms of staining intensity within ME in digitized images of tissue sections by densitometric image analysis (CellProfiler) and are now applying it to this study. With the second method, up to 10 epitopes can be quantified in a tissue section by transferring proteins from the section to a stack of 10 specialized membranes. This method will be particularly valuable for determining if there is a correlation between levels of 4HNE and proteins that are known targets of electrophiles. Confirmation of the presence of 4HNE in ME of women in our high BC incidence population led us to begin to test the feasibility of using proteomics technology to test whether chronic OXS in breast parenchyma is associated with changes in amounts and profile of reversibly oxidized proteins.

Conclusions: The study confirms our original observation suggesting that ME of most women in our high BC risk population is subject to chronic OXS. It underscores a need to develop methods for exploiting the vast archives of breast tissues for retrospective cytometric and molecular biological studies in order to identify changes leading up to the establishment of BC and to develop preventive strategies.

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P13-5: MICROBIAL EXPOSURES AND RISK OF POSTMENOPAUSAL BREAST CANCER: A POPULATION-BASED CASE-CONTROL STUDY

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The "hygiene hypothesis," the idea that reduced or delayed exposures to important microbial inputs hamper the development of a healthy immune system in early life and the maintenance of such a system in adult life, has been well studied as it relates to allergy, asthma, autoimmune disease, and other disorders of immune function. Because (1) hygiene hypothesis-related diseases share many epidemiologic commonalities with breast cancer (BC) and (2) there is substantial cross-regulation between immune and hormonal pathways; we hypothesized that reduced or delayed exposures to microbes or their byproducts, especially in early life, are associated with increased risk of BC development. We are conducting a population-based case-control study to examine the association of self-reported markers of microbial exposures with postmenopausal BC.

This study interviews by telephone women ages 50–79 living in four diverse (urban and agricultural) Northern California counties recently diagnosed with primary invasive BC during the period 1/1/2004–9/31/2005 and an equal number of community women without breast cancer. Control subjects are ascertained using a novel, address-based sampling procedure, which involves (1) randomly selecting addresses from a list purchased from a marketing agency; (2) using internet resources to find telephone numbers for selected addresses; (3) recruitment through both mailed and telephone contact and (4) active consideration of participation bias through statistical weighting of participant distributions to target U.S. Census distributions. Our structured questionnaire inquired about established BC risk factors and direct and indirect measures of age-specific microbial exposures including history of selected viral, bacterial, and parasitic infections, histories of factors consistently associated with asthma and allergy including exposure to furry pets, residential characteristics (near farm, near animal pens), attendance at day care/school, other exposure to older siblings and other children, selected vaccinations and antibiotics, insect stings, and other factors relating to microbe or endotoxin exposure. The questionnaire is administered by trained interviewers in English or Spanish and buccal cells are self-collected by participants.

As of January 2008, we have successfully completed interviews with 370 cases and 180 controls, yielding current response rates of 59% in cases and 30% among eligible controls. Control recruitment activities are continuing to recruit sufficient numbers

for appropriate quantification and adjustment for participation bias. Preliminary case-control comparisons will be presented. This and other efforts to identify new etiologic pathways for BC subtypes are needed in the absence of effective primary prevention. Furthermore, understanding how "hygiene hypothesis"-related exposures might explain urban/rural, socioeconomic, or racial/ethnic variation in BC incidence could provide novel means for addressing these disparities.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0283.

P13-6: ABSTRACT WITHDRAWN

P13-7: TOTAL XENOESTROGEN BODY BURDEN IN RELATION TO MAMMOGRAPHIC DENSITY, A MARKER OF BREAST CANCER RISK

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Background: Humans are exposed to a large number of chemicals in the environment that have estrogenic activity ("xenoestrogens"). Epidemiologic evidence has indicated that breast cancer risk increases with exposure to pharmaceutical estrogens and greater endogenous estrogen levels resulting from reproductive, menstrual, and lifestyle factors. It remains unclear whether exposure to xenoestrogens is also sufficient to increase breast cancer risk. Previous studies have investigated the relation between single xenoestrogen chemicals and breast cancer risk with inconclusive evidence regarding an association. The recent development of a technique to measure total xenoestrogen burden (i.e., the sum estrogenic activity of all xenoestrogens to which one is exposed) in biological samples presents a novel opportunity to better evaluate total xenoestrogen exposure in relation to breast cancer risk.

Hypothesis: Mammographic density is sensitive to estrogen exposure and a strong intermediate marker of breast cancer risk. We hypothesize that women with higher serum xenoestrogen levels will have greater mammographic density.

Specific Aims: This project will (1) characterize the distribution of total xenoestrogen burden and identify important sources of xenoestrogen exposure among a clinic-based sample of postmenopausal women and (2) evaluate the association of total xenoestrogen exposure with mammographic density, a strong intermediate marker of breast cancer risk.

Study Design: To accomplish these aims, 200 healthy postmenopausal women receiving their regularly scheduled screening mammogram at the University of Wisconsin Health West-Madison Clinic will complete a questionnaire regarding potential sources of xenoestrogen exposure, including diet, occupation, and lifestyle factors. Information will also be collected on the questionnaire regarding menstrual, reproductive, lifestyle, and medical history factors associated with mammographic density. Blood samples will be collected for the analysis of total serum xenoestrogen burden by the Wisconsin State Laboratory of Hygiene. Mammographic density as a continuous linear function will be measured from participants' mammograms using a computer-assisted method. Statistical analyses will be used to identify important predictors of total xenoestrogen burden and to measure the association between total xenoestrogen burden and mammographic density.

Impact: We have assembled a multidisciplinary team of clinical, laboratory, and population scientists to conduct an efficient investigation of the relation between xenoestrogen exposure and breast cancer risk in healthy women unbiased by the potential effects of tumor metabolism or breast cancer treatment on xenoestrogen levels. Since xenoestrogen exposure in humans is widespread, even a small association between total xenoestrogen exposure and mammographic density could be an important component of breast cancer risk. This study will quantify the magnitude of this association and identify sources of exposure that can be targeted for intervention.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0522.

P13-8: MATERNAL DIABETES AND BREAST CANCER IN DAUGHTER

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A growing line of evidence supports the developmental origins of cancer, in particular breast cancer. Numerous studies have associated high birthweight with an increased risk of breast cancer among premenopausal women. The diabetic intrauterine environment fosters a high birthweight. Moreover, diabetic mothers expose their fetus to elevated levels of glucose and insulin, which may promote cell proliferation. No data are available on the association between maternal diabetes and breast cancer risk in the daughter.

Using data from registries in Sweden linking the Multigeneration Register, Inpatient Registry, Death Registry, Emigration Registry, and Cancer Registry, we are establishing a retrospective cohort of women with diabetes and will assess the incidence of breast cancer in their daughters. We will study whether maternal diabetes increases the risk of breast cancer in the daughter and whether any association between maternal diabetes and an increased incidence of breast cancer is restricted to premenopausal breast cancer.

Our approach contributes to understanding the steadily increasing incidence of breast cancer especially among younger women and given the explosive increase in obesity and type 2 diabetes worldwide, any effect on the daughter's breast cancer risk may lead to a notable increase in breast cancer incidence in the near future.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0557.

P13-9: TELOMERE LENGTH, PERCEIVED STRESS, AND URINARY STRESS BIOMARKERS IN A NATIONAL STUDY OF MIDDLE AGE AND OLDER WOMEN

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Background: Telomeres are repetitive DNA sequences that cap and protect chromosomal integrity. Telomeres shorten with age, and short telomeres may lead to cellular senescence or carcinogenic transformation. Previous findings of shorter telomeres in mothers of chronically ill children suggest a novel link between psychosocial stress and cancer risk.

Methods: This cross-sectional study examined telomere length in relation to current perceived stress and urinary stress hormones in a sample (n=677) of women enrolled the NIEHS Sister Study, a prospective cohort of women ages 35–75 with at least one sister with breast cancer. Average leukocyte telomere length was determined by quantitative PCR. Current stress was assessed using the perceived stress scale and creatinine-adjusted neuroendocrine hormones in first morning urines. Age-adjusted linear regression models estimated differences in base pairs (bp) associated with stress measures and covariates.

Results: Telomere length decreased with increasing age (-26 bp per year). Shorter telomeres were also associated with current smoking, higher BMI, lower education, current poor health, and cardiovascular or metabolic disease. Women with perceived stress scores above the median (2 points out of a possible range of 0–16) had somewhat shorter telomeres (-113 bp, 95%CI -271, 44) than those at or below the median, although telomere length did not decrease monotonically with increasing stress score. Differences associated with perceived stress were significant among women age 55 and older (-283 bp; 95%CI -500, -32) and in those with higher than average urinary epinephrine (-422 bp; 95%CI -641, -202). These associations were not explained by differences in health or other covariates. Degree of familial breast cancer risk (number of or early age at onset of affected first-degree relatives) was not related to telomere length and did not modify the association of perceived stress and telomere length.

Conclusions: The overall association of stress and telomere length was modest in this cross-sectional study of women with relatively low perceived stress. Results for older women and those with higher epinephrine levels, however, suggest the effects of stress on telomere length may accumulate over time and vary depending on the biological stress response. Effects in these groups were equivalent to 10 or more years of age-related change and of similar magnitude to differences seen with cardiovascular/metabolic disease and current smoking. Longitudinal data may help determine

whether exposure to chronic or severe stressors affects telomere attrition and, in turn, variation in cancer risk.

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P13-10: DETERMINANTS OF WEIGHT GAIN IN WOMEN WITH EARLY STAGE BREAST CANCER

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Weight gain after breast cancer diagnosis is very common, occurring in 50%–95% of early stage patients undergoing adjuvant chemotherapy, and has been associated with poorer prognosis in several large studies, including the Nurse's Health Study. Previous explorations into the causes of weight gain in breast cancer survivors have been limited by use of retrospective study designs, which has limited the assessment of potential contributors to weight gain and has dealt primarily with treatment-related factors and clinical determinants. To date, prospective studies have been limited by relatively small sample sizes (N<100) and brief durations of follow-up because of the time-intensive nature of such studies. The aim of this study is to comprehensively examine why women gain weight after breast cancer diagnosis in a larger study, which includes examination of hormonal and inherited factors, changes in energy balance, and the potential impact of psychosocial factors. Since sex hormones and glucocorticoids regulate body weight and adipose tissue distribution, we hypothesize that treatment-related changes in sex hormone and cortisol levels play a role in treatment-induced weight gain and that complex interactions exist with genetic susceptibility, lifestyle, and psychosocial factors. The goals of the study are to examine post-diagnostic weight change in newly diagnosed breast cancer cases and (1) changes in sex hormone and cortisol levels; (2) genetic polymorphisms in sex hormone pathways; (3) energy intake, physical activity, and psychosocial factors; and (4) characteristics of the cancer and treatments received. We have initiated a prospective longitudinal study of weight gain in women ages 18 and older with nonmetastatic breast cancer (Stage I to IIIA). To date, we have enrolled 188 of the planned 290 participants with 112 women having completed their 6 months of follow-up and 67 women having completed 12 months of follow-up. We are collecting and banking serial biospecimens, which includes collection of serum, plasma, buffy coats, red blood cells, and overnight urine specimens. Waist and hip circumferences are measured, and all participants have their body composition determined by bioelectrical impedance, which allows for both whole-body and regional assessment of lean and fat mass. Participants also fill out an extensive self-administered questionnaire, which collects information on recent changes in menopausal status, occurrence, and severity of menopausal symptoms, level of physical activity, recent diet, appetite, and recent experience with fatigue, depression, and social support. These data are being collected at baseline and at 6 and 12 months and are currently being double entered into the study database and checked for data quality. We plan to evaluate the above factors in relation to weight and body composition changes during and following cancer therapy. This study will be the first to comprehensively examine predictors and modulators of postdiagnostic weight gain in women with breast cancer using a multidisciplinary approach encompassing hormonal changes, genetic polymorphisms, and psychosocial factors. The outcome of this research may shed light on why so many women suffer weight gain after breast cancer and will help guide the development of interventions targeting modifiable risk factors.

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RISK AND PREVENTION

Poster Session P14

P14-1: DNA METHYLATION AND BREAST CANCER RISK

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Background: One plausible mechanism for the environment to alter breast cancer susceptibility is through epigenetic effects on somatic cells, leading to activation or silencing of key genes in critical pathways. In addition to regional hypermethylation of specific regulatory and tumor suppressor genes, global DNA hypomethylation signals genome-wide aberrant DNA methylation. It is unknown whether DNA global hypomethylation measured in peripheral blood lymphocytes is related to breast cancer risk and whether environmental exposures that change over the life course are associated with global DNA hypomethylation.

Methods: We conducted two pilot studies of global hypomethylation measured in lymphocytic DNA using the methyl acceptance assay using the following designs: Study 1: To address Aim 1, we used compared sisters discordant for breast cancer from the New York site of the Breast Cancer Family Registry (BCFR); and Study 2: To address Aim 2, we used a prospective birth cohort study of women born between 1959–1966 at Columbia Presbyterian Hospital who were enrolled in the National Collaborative Perinatal Project. Study 1 includes 168 individuals (82 unique sets) ages 26–84. Study 2 includes 85 women ages 38–46. The second pilot study included participants from the New York Women's birth cohort who provided a blood sample (n=85). Global DNA hypomethylation was measured in lymphocytic DNA using the methyl acceptance assay; higher values with this assay indicate more demethylation or more global hypomethylation. Age-adjusted logistic regression was used to assess the association between global DNA methylation and breast cancer risk. Age-adjusted linear regression was used to examine the association between environmental exposures and global DNA hypomethylation.

Results: Study 1: Global hypomethylation was associated with a 50% increase in breast cancer risk (Quartile 4 relative to Q1: Odds Ratio (OR) = 1.5, 95% Confidence Interval (CI) = 0.6–3.7). This association was stronger when we restricted our analysis to cases with blood specimens collected within 2 years of diagnosis (Q4 relative to Q1 OR= 4.6 95% CI = 0.7–28.8). Study 2: Prenatal smoke exposure (p=0.07), birth length (p=0.03), parity (0.06), and age at first birth in adulthood (p= 0.02) were all associated with global DNA hypomethylation.

Conclusions: These pilot data, if replicated, suggest that measures of global hypomethylation may be important to cancer causation and may be associated with both early life and adult exposures.

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P14-2: CHEMOPREVENTIVE EFFICACY OF CURCUMIN IN AN IN VITRO MODEL FOR HER-2-POSITIVE, ESTROGEN RECEPTOR-NEGATIVE DCIS BREAST CANCER

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Introduction: Overexpression of HER-2 occurs in more than 60% breast ductal carcinoma in situ (DCIS), including ~30% estrogen receptor negative, chemotherapy-resistant, high-grade tumors with metastatic phenotype. An inverse correlation between expression of HLA class-I and HER-2 indicates loss of anti-tumor immune surveillance. Curcumin (CUR), the active component of spice Turmeric, is known for its immunomodulatory, antitumorigenic, and antitumorogenic properties.

Materials and Methods: Experiments were conducted utilizing our established human preclinical in vitro model for comedo DCIS characterized by ER⁺/PR⁺, HER2⁺ in 184-B5/HER cells.

Results and Discussion: 184-B5/HER cells exhibit hyperproliferation, shorter doubling time, higher anchorage-independent growth, and tumorigenicity. Our microarray data showed 260 differentially expressed probes (141 decreased, 119 increased) in these cells. Among the decreased signals, 45 belonged to IFN- γ /STAT-1 signaling pathway: STAT-1 (-4.43), IFI-27 (-25.8), ISGF-3G (-3.42), GIP3 (-7.46), OAS1 (-6.13), OAS2 (-5.27), OAS3 (-2.7), TAP1 (-1.94), MX1 (-12.29), IRF-7 (-5.41), and HLA class-I molecules; HLA-B (-2.01) and HLA-C (-2.03). Cell cycle analysis of 184-B5/HER cells exposed to 20 μ M CUR showed cytostatic arrest of growth, 85% inhibition of colony formation, decreased G₀/G₁:S+G₂/M ratio and increased apoptosis. 184-B5/HER cells treated with 10 and 20 μ M CUR showed 26.7% and 61.4% decreased Bcl-2 protein, and 12% and 35% increased Bax protein. The genomic profiles showed 557 modulated probes (177 upregulated, 380 downregulated). Among the downregulated cell cycle genes, 13 were specific to S+G₂/M phases: cyclin A1 (-2.79), cyclin A2 (-3.60), cyclin B1 (-3.45), cyclin B2 (3.89), CDK1 (3.96), CDK 2 (-1.69), Dbf4 (-2.56), Wee (-1.6), Cdc25C (-3.80), Chk1 (-2.58), BubR1 (-2.82), PTTG (-2.77), apoptosis-specific survivin (-3.28) besides NFkB (-1.86), and TOP2a (-5.94). We did not observe altered mRNA levels of the 42 IFN- γ related genes in 184-B5/HER cells, indicating effect of CUR via increase of HLA-I at the level of post-transcriptional regulation.

Conclusions: Treatment with curcumin shows preventive efficacy in this model of DCIS via cytostatic arrest of cells in S and G₂/M phases of cell cycle, inhibiting activation of ERK and induction of apoptosis. Curcumin treatment also leads to inhibition of inflammatory cytokines. These effects were not observed in the HER-2 negative 184-B5 cells.

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P14-3: MAMMARY CANCER CHEMOPREVENTION WITH THE POLYPHENOL RESVERATROL

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Despite recent advances in therapeutic treatments, breast cancer remains a serious disease and a leading killer among female cancers. In 2007, there were more than 200,000 new cases of breast cancer and 40,000 deaths. There is, and should be, a strong effort to work toward the prevention of this disease. It is now well accepted that environmental factors, especially diet and lifestyle, play a critical role in determining one's risk for breast cancer. One dietary polyphenol that has received much attention for its health benefits, including anti-cancer properties, is resveratrol, a phytoalexin found in red grapes and red wine. We hypothesized that resveratrol given in the diet would protect against chemically induced mammary cancer through mechanisms that involve mammary gland differentiation and epithelial cell proliferation and apoptosis. Female Sprague Dawley rats were treated with 1,000 mg resveratrol/kg AIN-76A diet from birth throughout life. At 50 days postpartum, animals were sacrificed to evaluate mammary whole mounts, cell proliferation, and apoptosis or treated with 60 mg dimethylbenz[a]anthracene (DMBA)/kg body weight to induce mammary adenocarcinomas. A follow-up tumor study with a lower dose of resveratrol (333 mg resveratrol/kg diet) was also done in the same manner. Both doses of resveratrol (1,000 mg and 333 mg) were able to suppress mammary tumor multiplicity and increase tumor latency in a statistically significant manner. Mammary whole-mount analysis revealed a modest increase in lobular structures, the least susceptible mammary terminal ductal structures to carcinogens. Cell proliferation and apoptosis assays revealed a significant decrease in mammary epithelial cell proliferation and a significant increase in apoptosis in mammary terminal end buds, the most susceptible structures to carcinogenesis in the mammary gland. As to the molecular mechanisms through which resveratrol may be protecting against mammary carcinogenesis and modulating mammary epithelial cell proliferation and apoptosis, we employed TaqMan low density arrays. We selected genes with a known role in mammary epithelial cell proliferation, apoptosis, steroid metabolism, and growth factors involved in normal and malignant mammary gland development. Exposure to resveratrol in the diet resulted in a significant decrease in gene expression of COX-1 and tended to increase the levels of PPAR- γ . Both of these genes can play a role in cell proliferation and apoptosis. We also observed a significant increase in the gene expression levels of caspases 2, 3, and 9, all of which are involved in the apoptotic cascade and may help to explain the increased apoptosis that we observed with resveratrol in the mammary gland. We conclude that resveratrol in the diet can protect against mammary carcinogenesis by modulating mammary gland differentiation and epithelial cell proliferation and apoptosis.

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P14-4: INHIBITION OF HISTONE DEACETYLATION MAKES MDA-MB-231 BREAST CANCER CELLS RESPONSIVE TO VITAMIN D

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Previous studies from our laboratory have shown that estrogen and progesterone receptor positive MCF-7, BT474 and T47D breast cancer cells respond to vitamin D metabolites such as 25-hydroxyvitamin D3 [25(OH)D3], 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], or analogs such as 1 α -hydroxyvitamin D5. The steroid receptor negative MDA-MB231 cells do not exhibit antiproliferative activity in response to vitamin D even though they express vitamin D receptors (VDR) and the expression is upregulated in response to vitamin D. We hypothesized that the histone deacetylation may prevent the VDR from accessing target gene promoters, resulting in the lack of antiproliferative activity. Incubation of MDA-MB231 cells with 15nM of Trichostatin A (TSA) or 25(OH)D3 (250nM) alone had minor effect on cell proliferation as measured by MTT assay. However, a combination treatment of TSA and 25(OH)D3 resulted in inhibition of cell growth by >60%. Previous studies in the literature as well as from our laboratory have shown that the antiproliferative effects of vitamin D are accompanied with increased expression of VDR and CYP24, therefore we evaluated the effects of TSA and vitamin D singly and in combination on the mRNA expression of VDR, CYP24, and CYP27B1 in these cells using quantitative RT-PCR analysis. Results showed that TSA alone inhibited the expression of CYP24, 25(OH)D3 alone enhanced CYP24 expression by 115-fold, whereas the combination

of TSA and 25(OH)D3 enhanced expression of CYP24 by 137-fold. These results indicate that inhibition of histone deacetylation by TSA in these cells make them responsive to 25(OH)D3. We expanded these studies to determine if any of the known chemopreventive agents derived from natural products can serve as histone deacetylation inhibitors in these cells. We evaluated effects of epigallocatechin gallate, sulforaphan, diallyl disulfide and butyric acid. However, none of these agents were comparable for the histone deacetylation inhibiting properties of TSA. These results indicate that inhibition of histone deacetylation for VDR positive vitamin D non-responsive cells may provide useful strategy for vitamin D responsiveness.

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P14-5: SUPPRESSION OF TUMOR FORMATION BY TREATMENT WITH A COX-2 INHIBITOR AND A PPAR γ AGONIST IN A MOUSE MODEL OF SPONTANEOUS BREAST CANCER

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Activation of cyclooxygenase-2 (COX-2) and inhibition of peroxisome proliferators-activated receptor-gamma (PPAR γ) have been observed in human and animal models of breast cancer. Both inhibition of COX-2 and activation of PPAR γ can inhibit proliferation of breast cancer cells in vitro. Since the downstream product of COX-2 is the PPAR γ agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2, treatment with both a COX-2 inhibitor and a PPAR γ agonist may be more effective than a COX-2 inhibitor alone. In this study we have examined the effects of the COX-2 inhibitor Celecoxib and the PPAR γ agonist F-L-Leu on mouse breast tumor cells in vitro and in vivo using C3 (1) SV40 T-antigen mice that spontaneously develop mammary adenocarcinomas.

We first created and characterized a mouse mammary adenocarcinoma cell (MMAC1) line derived from C3 (1) SV40 T-antigen mice. This line expresses both COX-2 and PPAR γ mRNA. In vitro, these cells are sensitive to both Celecoxib (IC₅₀ 79 μ M) and F-L-Leu (IC₅₀ 242 μ M). Addition of both 50 μ M Celecoxib (10% growth inhibition) and 80 μ M F-L-Leu (15% growth inhibition) resulted in a 70% inhibition of growth indicating that these compounds act synergistically to inhibit MMAC1 growth in vitro. To study the in vivo effects of these compounds, C3 (1) SV40 T-antigen mice (30 per arm) were given either control diet, diet containing either 1500 ppm Celecoxib, 750 ppm Celecoxib, 375 ppm Celecoxib, 125 ppm F-L-Leu, 62.5 ppm F-L-Leu, or 375 ppm Celecoxib and 62.5 ppm F-L-Leu. Mice were then followed for tumor formation up to 1 year. We found that the median age of death due to mammary tumors was significantly delayed in all of the treated groups compared to the untreated controls (mean age of tumor-related death 162 versus 252 to >300 days). As a single agent Celecoxib was slightly more effective than F-L-Leu at delaying tumor formation, but the combination of F-L-Leu and Celecoxib was significantly more effective than either single agent alone. We conclude that in C3 (1) SV40 T-antigen mice both Celecoxib and F-L-Leu are effective at delaying mammary tumorigenesis and that the combination of both agents is significantly more effective than either agent alone. Our findings suggest that a combination of low doses of COX-2 inhibitors and PPAR γ agonists may be useful in preventing breast cancer in high-risk populations.

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P14-6: THE t10,c12 ISOMER OF CONJUGATED LINOLEIC ACID (CLA) INDUCES APOPTOSIS IN MAMMARY TUMOR CELLS THROUGH AN ATYPICAL ENDOPLASMIC RETICULUM STRESS RESPONSE

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CLA inhibits rat mammary carcinogenesis in part by inducing apoptosis of preneoplastic and neoplastic mammary epithelial cells. The current study focused on the mechanism by which apoptosis is induced. In TM4t mammary tumor cells, t10,c12-CLA induced proapoptotic CEBP-homologous protein (CHOP) concurrent with the cleavage of PARP. Knockdown of CHOP attenuated t10,c12-CLA-induced apoptosis. Furthermore, t10,c12-CLA induced cleavage of ER-resident caspase-12. A selective inhibitor of caspase-12 significantly alleviated t10,c12-CLA-induced apoptosis. Together, these data suggest that t10,c12-CLA induces apoptosis through ER stress. To further explore the ER stress pathway, we examined the expression of the follow-

ing upstream ER stress signature markers in response to CLA treatment: XBP1 mRNA (unspliced and spliced); phospho-eIF2 α , ATF4, and BiP proteins. We found that t10,c12-CLA induced expression and splicing of XBP1 mRNA as well as phosphorylation of eIF2 α . In contrast, ATF4 was induced modestly, and BiP was not altered by t10,c12-CLA. Since the expression of BiP is usually increased during the ER stress response, our data suggest that t10,c12-CLA may induce an atypical ER stress response, using some, but not all, pathways of the ER stress response to induce apoptosis. In addition, using electron microscopy, we observed that t10,c12-CLA treatment resulted in marked dilatation of the ER lumen, supporting induction of ER stress by t10,c12-CLA. The above effects were specific to t10,c12-CLA since the c9,t11-CLA isomer did not show the same effect in TM4t mammary tumor cells despite the fact that both isomers are equally efficacious in some in vivo mammary cancer models. In summary, our data demonstrate that t10,c12-CLA induces apoptosis of mouse mammary tumor cells and that the apoptosis is mediated, at least in part, through an atypical ER stress response that culminates in the induction of CHOP and cleavage of caspase-12.

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P14-7: STANDARDIZATION OF IMMUNOHISTOCHEMICAL STAINING ON NORMAL, PROLIFERATIVE, AND ATYPICAL HUMAN BREAST TISSUE

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We have previously shown that the somatostatin analog, SOM230, can inhibit IGF-I activity in normal mammary tissue of hypophysectomized and oophorectomized female rats. As IGF-I is essential for both estrogen and progesterone action on the mammary gland, we hypothesized that this compound might be a useful agent for the prevention of breast cancer instead of estrogen antagonists. We are in the process of doing a "proof-of-principal" study in women who have breast lesions that put them at higher risk for breast cancer. Breast biopsies that show such lesions will be analyzed for cell proliferation, apoptosis, and IGF-I induction of phosphorylation of IRS-1 and compared with clinically necessary excisional breast biopsies after 10 days of treatment with SOM230. The expected result would be a decrease in cell proliferation and IRS-1 phosphorylation and an increase in apoptosis after treatment with SOM230.

In preparation for these studies, we have evaluated the similarities and differences in proliferation and apoptosis in normal human tissue and proliferative lesions (sclerosing adenosis and usual ductal hyperplasia). Patients with proliferative lesions have a somewhat higher relative risk (1.88) of developing breast carcinoma.

The mean percentage of Ki67 positive proliferating cells in 7 samples of proliferative breast lesions was 8.06% (range 3.15 to 19.25) compared to 7 samples of normal breast (terminal ductal lobular units), which had a mean proliferation rate of 1.92 % (range: 1.27 to 2.73; $p < 0.02$). In contrast, the rate of apoptosis was lower in the hyperplastic lesions than the normal tissue (0.57% versus 1.54%, respectively; $p < 0.001$). Taken together with our preclinical data in rats, these findings in human tissue suggest that hyperplastic cells may be more sensitive to the effects of estrogen and IGF-I than non-hyperplastic epithelial tissue. Whether IGF-I inhibition by SOM230 will inhibit formation of hyperplastic lesions in women at high risk for breast cancer by inhibiting cell proliferation and stimulating apoptosis, as we have seen in rats, must await clinical data from our ongoing study.

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P14-8: NOVEL SULINDAC SULFIDE DERIVATIVES FOR BREAST CANCER CHEMOPREVENTION

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Nonsteroidal anti-inflammatory drugs (NSAIDs) have potential chemopreventive indications for breast cancer. Unfortunately, toxicity resulting from cyclooxygenase (COX) inhibition limits their clinical utility. Recent studies suggest that the mechanism for the anti-cancer activity of NSAIDs may involve a non-cyclooxygenase (COX) target. For example, the sulfone metabolite of the NSAID, sulindac, does not inhibit COX-1 or -2, yet inhibits tumorigenesis in various experimental models, including mammary tumorigenesis. Sulindac sulfone appears to selectively induce apoptosis by inhibiting phosphodiesterase (PDE) isozymes that cause an elevation of the second messenger, cyclic GMP. Sulindac sulfone, however, has weak potency, and the specific PDE isozyme(s) responsible for its activity is, as yet, unidentified. We have recently prepared a new amide derivative of sulindac sulfide, which lacks COX-1 and COX-2 inhibitory activity, yet shows significantly enhanced growth inhibitory potency compared to sulindac sulfone and sulfide against a variety of tumor cell lines, including several derived from breast adenocarcinomas. These data suggest that new, more potent anticancer agents based on the sulindac sulfide scaffold can be designed that have potential for minimal COX-dependent toxicity. We hypothesize

that it is feasible to develop safer and more potent drugs for the prevention and treatment of breast cancer by chemically modifying sulindac sulfide to block COX inhibition while enhancing potency to exert greater in vivo efficacy. Our program involves the rapid, parallel synthesis and evaluation of approximately 340 new derivatives of sulindac sulfide to optimize a prototypic agent, SRI 21009. Compounds are evaluated for growth inhibitory effects against three human breast tumor cell lines and for inhibitory effects on COX and PDE enzyme activity. Those analogs that show potent growth inhibitory activity with minimal COX-1 or COX-2 inhibitory activity will be identified by in vitro screening. The most active agent(s) will also be used as molecular probes to identify the target PDE isozyme. Finally, a lead agent will be selected for in vivo efficacy assessment in a murine xenograft model using optimally responsive human breast tumor cell line. Sulindac sulfide and sulfone will be used as in vitro controls to assess the feasibility of uncoupling COX inhibition from growth inhibition and as benchmarks for improving safety and efficacy. Sulindac will be used as a control for in vivo studies. Through the optimization of more potent and selective sulindac sulfide derivatives that lack COX inhibition, it may be possible to develop safer and more efficacious drugs for breast cancer chemoprevention with potential for therapeutic applications.

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P14-9: HUMAN BREAST TISSUE BIOAVAILABILITY OF TOPICALLY APPLIED LIMONENE

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Limonene, a monocyclic monoterpene, is a major component in the essential oils of citrus fruits. Oral feeding of limonene has been shown to increase tumor latency during the initiation and promotion stages of rat mammary carcinogenesis. However, the effective oral dose determined in rodent mammary cancer models extrapolates to a human dose that is not feasible for long-term oral consumption. Chemically, limonene is highly lipophilic. It has been long explored as an enhancer for percutaneous absorption of pharmaceutical drugs, including tamoxifen, by interacting with intercellular stratum corneum lipids to increase diffusivity. In vitro studies have also shown that limonene can penetrate through human skin. With its high lipophilicity, limonene has favorable tissue distribution and distributes preferably to adipose and mammary tissues in rats after oral administration.

We hypothesize that topical application of limonene to the breast can achieve high limonene levels in the breast tissue and exert regional biological activities that could lead to breast cancer prevention. The overall objective of the proposed research is to conduct a clinical study in healthy pre-menopausal women aiming to determine breast tissue bioavailability of limonene following topical limonene application to the breast. Because limonene is a major component of citrus essential oils, such as sweet orange oil or lemon oil, and these oils are used in aromatherapy massage, we propose to use limonene containing massage oil as a safe topical formulation in this exploratory study. Limonene levels in the nipple aspirate fluid and plasma collected after repeat application of diluted sweet orange oil will be determined using gas chromatography-mass spectrometry.

We received final regulatory approval of the clinical protocol and related documents in the fall of 2007. A commercially available organic sweet orange oil product has been selected for the study based on our laboratory testing of limonene content and impurities of six commercial products. The study plans to enroll a total of 36 women and has accrued 2 as of December 2007. An assay for quantification of limonene levels in plasma and nipple aspirate fluid has been developed and is being validated for clinical application.

The proposed study is one of the first to evaluate the clinical feasibility of localized delivery of a breast cancer preventive agent. If successful, other defined topical formulations can be developed and clinical studies can be conducted to determine the clinical activities of topically applied breast cancer preventive agents. We believe that this novel approach could increase drug efficacy in the breast and reduce systemic toxicity.

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P14-10: PILOT STUDY OF ROSIGLITAZONE THERAPY IN WOMEN WITH BREAST CANCER: EFFECTS OF SHORT-TERM THERAPY ON TUMOR TISSUE AND SERUM MARKERS

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Introduction: Peroxisome proliferator-activated receptor gamma (PPAR γ) is a steroid nuclear receptor that is activated by natural compounds such as specific fatty acids and synthetic drugs such as thiazolidinedione antidiabetic agents. Expressed in nor-

mal and malignant mammary epithelial cells, activation of PPAR γ is associated with antiproliferative effects on human breast cancer cells in preclinical studies. The purpose of this study was to test the hypothesis that PPAR γ ligand therapy might inhibit tumor growth and progression in human breast cancer.

Experimental Design: We conducted a pilot trial of short-term (2 to 6 weeks) treatment with the thiazolidinedione rosiglitazone in 38 women with early stage (Tis-T2, N0-1, M0) breast cancer, administered between the time of diagnostic biopsy and definitive surgery.

Results: Short-term treatment with rosiglitazone (8 mg/day) did not elicit significant effects on breast tumor cell proliferation, using Ki67 expression as a measure of cell proliferation and surrogate marker of tumor growth and progression. In pre-treatment tumors notable for nuclear expression of PPAR γ by immunohistochemistry, down-regulation of nuclear PPAR γ expression occurred following rosiglitazone administration ($p=0.005$). No PPAR γ mutations were identified, and the incidence of P12A and H446H polymorphisms did not differ relative to U.S. controls ($p=0.5$). Treatment with rosiglitazone resulted in increased serum adiponectin ($p<0.001$), decreased insulin levels ($p=0.005$), and increased insulin sensitivity ($p=0.004$). Rosiglitazone was well-tolerated without serious adverse events.

Conclusion: Our data indicate that short-term rosiglitazone therapy in early stage breast cancer patients leads to local and systemic effects on PPAR γ signaling that may be relevant to breast cancer.

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P14-11: DEVELOPMENT OF (-)-GOSSYPOL-ENRICHED COTTONSEED OIL, (-)-GPCSO AS A POTENT ANTI-CANCER FOOD COMPONENT FOR HUMAN BREAST CANCER

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Gossypol (GP) is a naturally occurring polyphenolic pigment present in cottonseed. GP exists, naturally, as a racemic mixture of 35% (-)-GP and 65% (+)-GP. GP is also found in cottonseed products such as cottonseed oil (CSO) and cottonseed meal (CSM) that are consumed by humans and food-producing animals. In the United States, CSO, and thus GP, directly enters the human diet through its use in cooking, frying, food processing, and its presence in salad dressing, shortening, margarine, canned or snack foods and chewing gum.

GP has been shown to possess anticancer activity against a variety of cancers. We demonstrated that (-)-GP is a more potent chemopreventive agent than either racemic (\pm)-GP and (+)-GP. We also showed that CSO containing a high concentration of the (-)-racemate ((-)-GPCSO) exhibits potent anti-breast cancer activity than the conventional CSO. In collaboration with USDA researchers, our laboratory has access to a high (-)-GP (65%) and low (+)-GP (35%) cottonseed oil. The anti-breast cancer potency of (-)-GPCSO was evaluated in human breast cancer cell lines and primary cultured human breast cancer epithelial cells (PCHBCEC) in vitro. Using a non-radioactive cell proliferation assay, we found that (-)-GPCSO at levels of 0.025, 0.05, and 0.1% in culture medium (equivalent to 40, 80, and 160 nM of GP) significantly inhibited the proliferation of ER α positive MCF-7 cells at 21, 34, and 71%, and ER α negative MDA-MB-231 cells at 39, 46, and 50%, multidrug resistant MCF-7 Adr cells to 27, 58, 79% and PCHBCEC at 4.5, 31, and 62%, respectively. Concentrations of (-)-GPCSO at 0.05, 0.1, and 0.2% significantly suppressed the mRNA expression levels of cyclin D $_1$ to 19, 23, and 31% in MCF-7 cells and to 21, 66, and 81% in PCHBCEC, respectively. Our in vitro experimental data indicates that (-)-GPCSO is a potent anti-breast cancer bio-active food ingredient. Continued research and development of (-)-GPCSO as a low cost and abundance chemopreventive food component for cooking, salad dressing, and processed foods for practical application to general population or the population with high breast cancer risk are warranted.

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P14-12: VALIDATION OF A PRECLINICAL MODEL FOR THE INVESTIGATION OF MENARCHEAL AGE ON BREAST CANCER RISK

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Women with late menarche have breast cancer rates approximately 2-fold lower than individuals with early menarche; however, the mechanism of protection is unknown. One theory suggests that late menarche is associated with persistent qualitative differences in the hormone axis. We investigated the hypothesis that early and late sexual maturation differ in estrous cycle regularity or systemic estradiol levels. Two hundred

20-day old female SD rats were monitored for vaginal opening (VO). Thirty-nine rats had VO between 28 and 30 days of age, 123 rats had VO between 31 and 33 days of age, and 38 rats had VO at 34 days of age or later. For this study, rats with vaginal opening at 30 days of age or younger were utilized for the early VO group and those with vaginal opening of 34 days of age or later for the late VO group.

To characterize whether relationships exist between age at VO and adult body weight, or between age at VO and weight at VO, body weights were recorded twice weekly. As a group, rats with early VO were ~9% heavier than the rats with late VO, differences in weight started at 28 days of age and persisted into adulthood. These data model the relationship observed between onset of puberty and body weight in women. However, when weight of each rat on the day of VO was evaluated, it was found that rats in the early VO group reached sexual maturation at a much lower body weight than rats in the late VO group. These data suggest that body weight alone does not account for entry into VO. Rather, a combination of body weight in conjunction with a "permissive" endocrine profile is likely to be responsible for onset of VO.

The question of whether age at VO influences the length or regularity of the estrous cycle was determined over a 2-week period, starting at 63 days of age. No differences in length of cycle, number of days of estrogen stimulation, or cycle regularity were observed between groups. To determine whether circulating estradiol levels differ between early and late VO groups, blood was obtained from regular cycling rats during three stages of the cycle: proestrus (P), estrus (E), and diestrus 1 (D1) with an N=14 in the early VO group and an N=12 in the late VO group. Surprisingly, at all three stages, circulating estradiol levels were lower in rats with early VO (6.0, 4.3, and 4.2 pg/ml in P, E, and D1, respectively) than in rats with late VO (7.6, 5.5, and 5.4 pg/ml).

Mammary tissue was harvested from rats during estrous from rats injected with 50 mg BrdU/Kg body weight 2 hr prior to tissue harvest. The questions of whether the differences in circulating estradiol levels observed between groups corresponded to differences in gland morphology or MEC proliferation were evaluated. Based on morphometric analyses, a trend toward decreased cellularity in the early VO group was observed. IHC detection of BrdU corroborated the decreased cellularity data, as early VO rats had an average MEC proliferation rate of 5.78% and late VO rats had average MEC proliferation rate of 8.09%. These preliminary data indicate that early VO is characterized by low body weight, a modest decrease in circulating estradiol levels and a reduced proliferative response in the mammary gland. The significance of these observations remains to be determined. Once characterized, this preclinical model may prove useful to investigate the mechanism(s) by which early menarche increases breast cancer risk.

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P14-13: IDENTITY BY DESCENT MAPPING USING DENSE MARKER MAPS IN EXTENDED PEDIGREES

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Background: Our project is based on the following three observations:

1. Single nucleotide polymorphism (SNP) assays provide an efficient way of genotyping an individual along their complete genome using over 1,000,000 loci evenly spread throughout the genome. This density means that many existing statistical approaches are inappropriate as they assume marker linkage equilibrium (LE) while the dense SNP markers are in strong linkage disequilibrium (LD).
2. Extended families with an excess of women with breast cancer are a powerful resource for mapping susceptibility genes. Studies of such Utah families led to the discovery of the breast cancer susceptibility genes BRCA1 and BRCA2. There are still many families whose excess of cases cannot be explained by these two genes, and which may segregate other breast cancer susceptibility genes.
3. There is considerable work concerned with mapping genes by detecting genomic regions shared by individuals that are identical by descent (IBD) from a portion of a common ancestral chromosome. If the probability of cases sharing an IBD region purely by chance is sufficiently small, the region becomes a strong candidate for containing a susceptibility gene.

Methods: Our strategy for mapping breast cancer susceptibility genes is as follows:

- * To genotype distantly related breast cancer cases in extended high risk Utah breast cancer pedigrees.
- * To compute statistics that measure at which SNP loci cases are identical by state (IBS).
- * To use simulation to determine where the runs of high scores indicate that the observed IBS sharing may be due to underlying IBD sharing.

Progress: We have identified 3 pedigrees with tight clustering of breast cancer cases representing more than 20 meioses between all cases. Individuals from each family have tested negative for both BRCA1 and BRCA2 mutations and none have tested positive. DNA from 25 cases in these families has been submitted for genotyping and we are waiting for the results.

We have downloaded data on 60 unrelated Utah individuals from the HapMap project web site and extracted control allele frequencies. We have scanned the data for genomic regions of high allele sharing and identified anomalies on chromosomes 5 and 18. Had we not done this we could have been led to false positive conclusions in these regions.

In the area of LD modeling, we have submitted a paper (Thomas, A. 2008, Estimation of graphical models whose conditional independence graphs are interval graphs and its application to modeling linkage disequilibrium) that describes the effects of restricting the possible class of graphical models to the sub-class of interval graphs. This was shown to allow for more efficient model estimation and larger numbers of loci. Programs implementing this estimation process have been written and will be the basis of future simulation programs.

Impact: Our research will enable exploitation of recent advances in genotyping technology in family studies. This can lead to new gene discoveries that have eluded current methods and study designs. Our methods could provide a narrow localization for a breast cancer susceptibility gene that can then be exploited by mutation screening. The impact of a subsequent gene finding would likely be as significant as that of BRCA1 and BRCA2.

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P14-14: HUMAN PAPILLOMA VIRUSES (HPVS), FOOD, HORMONES, AND BREAST CANCER

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Hypothesis: We hypothesize that food consumption patterns determine circulating hormone levels that in turn promote the replication of the hormone-dependent oncoviruses—HPVs and mouse mammary tumor viruses (MMTVs), which may initiate breast carcinogenesis.

Background: The two most striking features of human breast cancer are (1) high prevalence in Western countries and lower prevalence in Asian countries (up to 6-fold difference—Table 1) and (2) high prevalence among females (an approx. 150-fold difference to males). Breast cancer prevalence increases following migration from low to high risk for breast cancer countries (Table 2). It follows that breast cancer must be mainly caused by external factors.

Food consumption patterns are the main determinants of circulating sex and other hormone levels, which in turn determine the age of menarche and menopause, birth weight, height plus weight gain in postmenopausal women. These are all risk factors for breast cancer. These factors are probably due to the influence of estrogens and other hormones (Yager et al. 2007).

HPVs and Breast Cancer: Different HPV types are the proven cause of cervical cancer and the probable cause of head and neck cancers (IARC 1995, D'Souza et al. 2007).

HPV replication is hormone dependent as is breast cancer (Webster et al. 2001).

HPV viral genetic material is identifiable in human breast tumors and cells but rarely in normal breast tissues. HPV high-risk types 16, 18, and 33 have been identified in breast tumors in 11 of 13 studies (Lawson et al. 2006).

HPVs malignantly transform breast epithelial cells due to the influence of the HPV E6 and E7 oncogenes that respectively bind and suppress the tumor suppressor genes p53 and pRb (Dimri et al. 2005).

HPVs are present in cancers occurring in human nipple milk ducts. These cancers have typical histological features of HPV-associated human cervical cancers (de Villiers et al. 2005).

The means of HPV transmission to the breast are not known. HPV-positive breast cancer occurs more commonly in younger women, accordingly, transmission of HPVs may be associated with sexual activity, as is the case with HPV-associated cervical and some oropharyngeal cancers (Lawson et al. 2007, D'Souza et al. 2007).

This evidence is supportive of a role for HPVs in the etiology of human breast cancer.

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P14-15: MOUSE MAMMARY TUMOR VIRUSES (MMTV) AND HUMAN BREAST CANCER

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MMTV is the established cause of mammary tumors in field and experimental mice. MMTV is transmitted both through mouse mother's milk and the germline. MMTV is influenced by hormones as is breast cancer. Estrogens induce mouse mammary tumors in the presence of MMTV but not in its absence [1]. Sequences related to hormone-responsive elements in MMTV-like DNA have been identified in human breast cancers [2]. MMTV-like envelope gene sequences have been identified in human breast cancers but rarely in normal breast controls in 10 of 13 studies conducted since 1995 [3]. The prevalence of MMTV *env* gene sequences increases with the progression from human breast hyperplasia, ductal carcinoma in situ to invasive carcinomas [4]. In situ PCR-based studies have demonstrated the location of MMTV-like *env* transcripts in breast cancer cells [5]. The almost complete proviral structure of MMTV-like virus has been identified from human breast carcinomas [6]. Seventy percent of the RNA from MMTV-like viral particles from human breast tumor metastases have been sequenced and shown to be 85% homologous to MMTV [7]. MMTV-like *env* sequences have been identified in 7 of 14 cultured human breast cancer cell lines [8,9]. Experimental infection of human breast cells by MMTV has been achieved [10]. Transformation of human breast cells by MMTV has been demonstrated [11]. An MMTV-like superantigen has been identified in human breast cancer [12]. We have shown that 42.4% (n=66) of human breast tumors have some similar histological characteristics to MMTV+ mouse mammary tumors [13]. p53 protein is more highly expressed in aggressive human breast tumors and is significantly more highly expressed in MMTV+ than MMTV- breast tumors [14]. Recently no MMTV-specific antibodies were identified in women with breast cancer [15]. This observation challenges the concept of MMTV as a possible cause of human breast cancer. An explanation may be the reduced immune response to MMTV by both human and mouse newborns [16].

Transmission of MMTV: Mouse-to-human transmission of MMTV is a possibility. Transmission via human milk is possible despite the apparent consistent evidence that there is no association between breast feeding and breast cancer. We have shown that virtually all newborn human infants are exposed to colostrum or milk soon after birth even if breast feeding is not established [17]. The only direct evidence is the observation of MMTV-like particles in human milk from women with breast cancer and women with a family history of breast cancer [18].

Overall the evidence is suggestive of a possible role for MMTV-like viruses in the etiology of breast cancer.

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P14-16: ARE SOME BREAST CANCERS SEXUALLY TRANSMITTED?

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Three studies report that the age of women with HPV-positive breast cancer is significantly younger than women with HPV-negative breast cancer. On average Greek women with HPV-positive breast cancer were 15 years, Australian women 8 years, and Canadian and Syrian women were 11 years younger than those with HPV-negative breast cancer (Kroupis et al., 2006, Lawson et al., 2006, Akil et al., 2007). Two studies report no difference in the age of women with HPV positive or negative breast cancer (Hennig et al., 1999, Damin et al., 2004) (Table 1).

Based on the younger age of some women with HPV-positive breast cancer and the higher incidence of HPV-positive cervical cancer among younger women with multiple sexual partners, we have hypothesized that high-risk HPV may be transmitted to the breast during sexual activities (Lawson et al., 2006).

The underlying assumption to these observations and hypotheses is that high-risk HPVs may have a causal role in some breast cancers.

High-risk HPVs, in particular HPV types 16, 18, 31, 33 and less commonly additional types, are the accepted cause of cervical and other ano-genital cancers which are transmitted by sexual activity among younger women with multiple sexual partners (IARC, 1995). Less well known is the likely causal role of high-risk HPVs in cancers of the head and neck which are also sexually transmitted (D'Souza et al., 2007).

The main means of transmission of HPVs is by surface contact. The human papilloma virions are released when the cornified envelope of cells desquamate (Bryan and Brown, 2001). The possibility of transmission of high-risk HPVs during sexual activity is demonstrated by the high prevalence of these viruses in male and female genital organs. The prevalence of high-risk HPVs in male genital organs is between 5 and 50%, semen 2 to 83%, and urine up to 7% varying between populations (Dunne et al., 2006). High-risk HPVs are also present in normal, benign hyperplastic, and malignant prostate tissues (Zambrano et al., 2002). The prevalence of high-risk HPVs in females varies between populations and dramatically so between age groups, with the prevalence in near normal cervical smears from UK women 61% at ages 20 to 24 decreasing to 14%–15% in those over 50 years (Cotton et al., 2007).

The evidence that high-risk HPVs may have an etiological role in some human breast cancers and that the means of transmission may be by sexual activity, is substantial, but far from conclusive.

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P14-17: ANALYSIS OF EPIGENETIC CHANGES INDUCED BY EPSTEIN-BARR VIRUS INFECTION IN BREAST CARCINOMA

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Epstein-Barr virus (EBV) is a human DNA virus that persistently infects over 90% of adults, usually without consequence. However, EBV is linked to a diverse array of lymphoid and epithelial malignancies originating at various anatomical sites. The association with any one tumor is often erratic, with only a fraction carrying the virus. It is within this context of incomplete association that we view the highly controversial detection of EBV in invasive breast cancers. Preliminary data from our laboratory suggest that transient EBV-infection of carcinoma cell lines in vitro can stably alter cell morphology and gene expression patterns so that the resultant phenotype is retained for generations after EBV DNA loss. Reversion to the parental phenotype on treatment with an inhibitor of methyltransferases suggests an epigenetic effect. We propose a model whereby EBV infection of tumor tissue alters the infected cell epigenetically so that EBV's continued presence would no longer be required to provide a selective advantage to the tumor cell. Our objectives are (1) to identify EBV-induced epigenetic modifications in breast carcinoma cell lines infected in vitro and (2) to determine whether such an epigenetic footprint induced by EBV can be identified in archival samples of breast carcinoma.

Transiently infected clones of the breast carcinoma cell line MDA-MB-231 were generated by passage of cells under G418 selection after in vitro infection with two neomycin recombinant EBV strains. Because in vitro infection of carcinoma cell lines is highly inefficient, a potential caveat is that EBV infected a cell possessing a particular epigenetic state. To avoid this issue, three 231 clones were infected. After 10 passages on selection, all cells were EBV-positive, and selection pressure was removed to allow for viral loss. Transiently infected, EBV-negative clones were established and are currently being analyzed for phenotypic and epigenetic changes. Once an EBV epigenetic footprint is identified in breast carcinoma cell lines, archival samples of breast tumors will be examined for similar epigenetic alterations.

Retention of a virally induced epigenetic imprint in transiently infected cells introduces a new paradigm for how a human tumor virus can mechanistically regulate a

cell in a “hit-and-run” fashion. Identification of such an epigenetic footprint characteristic of past EBV infection can be used to uncover EBV as a hidden co-factor to breast carcinogenesis. The importance of this research is that: (1) it may provide a mechanistic basis for EBV’s contribution to the progression of breast carcinogenesis that is consistent with its erratic viral association with breast cancer; (2) epigenetic modifications can be reversed by drugs currently in human trials; (3) EBV-induced gene silencing via epigenetic modifications may be applicable to other EBV-related malignancies or disease state; (4) identification of a viral “footprint” may allow EBV to be implicated in other disease states in the absence of detectable viral DNA or gene products; and (5) the finding potentially broadens the role of human tumor virus in carcinogenesis, including that of other agents with similar activity.

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P14-18: BOVINE LEUKEMIA VIRUS IN HUMAN BREAST EPITHELIUM IS A RISK FACTOR FOR BREAST CANCER

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We cannot hope to curb the current global epidemic of breast cancer without knowing its cause. Breast cancer in mice is caused by a milk-transmitted virus, MMTV, and provides a model for a viral etiology of human breast cancer. Since humans drink more cows milk than human milk, considering a bovine virus as a possible cause of human breast cancer is not unreasonable. Bovine leukemia virus (BLV), an oncogenic retrovirus, causes widespread infection of cattle globally. In 1-5% of infected cattle leukemia/lymphoma may develop, but most infected cattle remain healthy, are retained in the herd, and are sources of beef and dairy products. Infectious virus may be present in bovine foodstuffs not adequately cooked or pasteurized. In cattle, BLV infects lymphocytes and mammary epithelial cells. It infects goats and sheep naturally, and human cells in vitro. Evidence is accumulating that some humans are infected

with BLV. IgG antibodies to BLV were detected in 39% of human volunteers. BLV proteins and proviral DNA were detected in human leukocytes and breast epithelium.

The purpose of this case-control study was to determine whether BLV proviral DNA is present more frequently in the breast tissue of women with a diagnosis of breast cancer than in women who have never had breast cancer. This is a first step in determining whether BLV in breast tissue is a risk factor for developing breast cancer. Breast tissues were obtained through the Cooperative Human Tissue Network from four catchment areas: Philadelphia, PA, Columbus, OH, Birmingham, AL, and the San Francisco East Bay. Breast tissue sections were tested by in situ polymerase chain reaction (IS-PCR) for the presence of BLV proviral DNA using primers from the BLV tax region, which is not homologous to any region within the human genome. Results for 241 subjects completed to date indicate that the breast tissue of women with a diagnosis of breast cancer tested positive for BLV proviral DNA significantly more frequently than breast tissue from women with no history of breast cancer ($P < .0001$; age-adjusted odds ratio = 3.22, confidence interval = 1.7-6.2). Attributable risk was 39%, meaning that BLV might be the primary risk factor in as many as 39% of all breast cancer cases. Ductal carcinomas comprised 90% of the malignant specimens. There were not enough other types to determine if their frequency of BLV presence differed from that of ductal carcinomas. In 69% of the cases of paired malignant and “uninvolved” breast tissues from the same donor, BLV was present in the nonmalignant breast epithelium. This suggests that virus infection preceded malignant transformation, i.e., cancer may have been a rare, delayed event within a population of BLV-infected cells widespread in the breast (field effect), rather than virus infection being selective for cells already malignant. Our results suggest an association between infection of breast epithelium with BLV and development of breast cancer. If further studies support our data, and indicate how the virus is transmitted to humans, it may be possible to prevent some breast cancer cases by preventing or controlling human infection with BLV.

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DRUG DISCOVERY AND DEVELOPMENT I

Poster Session P15

P15-1: ENZYMATICALLY SYNTHESIZED POLYMERIC CATECHINS EXHIBIT POTENT AND SPECIFIC ANTI-PROLIFERATIVE ACTIVITY

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Background: Naturally occurring monomeric catechins (constituents of green tea and wine) are known to exhibit chemoprotective and therapeutic properties. Among these naturally occurring catechins, epigallocatechin gallate (EGCG) has been reported to be a potent inhibitor of human breast cancer cell proliferation in vitro at doses >20 μ M. However, EGCG loses activity within 1 hour of solubilization. Ironically, most drugs that are used to treat cancer are synthesized using multiple steps that involve the use of carcinogenic chemicals or generate carcinogens during production.

Objectives: The primary goal of this work was to develop a simple eco-friendly semisynthetic method to obtain polymerized forms of catechins that maintained bioactivity but were highly stable in aqueous solutions.

Methodology: Various stereoisomers of catechins were biocatalytically polymerized using the plant enzyme horseradish peroxidase as a catalyst in aqueous media. This one-pot oxidative polymerization was carried out in ambient conditions yielding water-soluble polymeric catechins. The starting materials, intermediates, and the products obtained were completely biocompatible and therefore do not have any harmful effects to human life/environment. Proliferation assays were performed using commercially available human cell lines MCF-7 (human, low-metastatic, +ER breast cancer cells), MDA-MB-231 (human, high-metastatic, -ER breast cells), and normal mammary epithelial cells (MCF12A). The ability of the polymerized forms of catechins to inhibit the growth of cancer cells was evaluated in a dose-dependent manner and compared with other catechins such as EGCG.

Results to Date: We found that polymerization significantly enhances the stability and efficacy of these materials as antiproliferative agents. Polymerized catechins were more effective at lower doses and more selective for cancer cells than any catechin monomers tested. In our studies, we found EGCG at 5 μ g/mL was a poor inhibitor of both the breast cancer cell lines, and it also growth inhibited normal mammary epithelial cells to the same extent as the cancer cells. In striking contrast, the growth of MCF-12A cells was not significantly affected when treated with the polymeric catechin at equivalent doses (5 μ g/mL to 0.1 μ g/mL). At these same doses, however, the polymeric catechin significantly growth inhibited the cancer cells using student T-test statistical comparison to controls. The polymerized catechin was stable for 3 months at room temperature.

Conclusions: Most drugs used to treat breast cancer are toxic to normal cells and are often produced using synthetic methods that involve ingredients or by-products that are carcinogenic. In our work presented here, we have shown that monomeric catechins have been polymerized using environmentally friendly methods, yielding active and stable products with selective antiproliferative activity for human breast cancer cells. It is envisioned that the synthesis of anti-cancer drugs from bio-based materials, using green methods, will cause a paradigm shift in the development and manufacturing of cancer drugs.

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P15-2: TARGETS FOR NOVEL BREAST CANCER THERAPIES: ESTROGEN-RELATED RECEPTOR α - CO-ACTIVATOR PROTEIN-PROTEIN INTERACTIONS

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Approximately 50% of all breast tumors grow independently of hormone, regardless of initial hormone receptor status; therefore, current hormonal therapies, such as tamoxifen and aromatase inhibitors, are not treatment options for these breast cancer patients. There is a great need for novel therapies for hormone-independent breast cancers. Estrogen-related receptor alpha (ERR α) may be a new therapeutic target since it possesses overlapping sequence and functional similarity to the hormone receptor ER α . For example, ERR α binds to estrogen response elements (EREs) in the promoters of a subset of estrogen-responsive genes, such as pS2, PgR, ErbB2, and others that are involved in cell proliferation and anti-apoptosis. Also, ERR α transcriptional activity is enhanced by the presence of co-activators. However, ERR α acts independently of hormone. Moreover, ERR α expression correlates with hormone receptor-negative breast tumors and with decreased disease-free survival of breast cancer patients. These observations strongly suggest that ERR α plays an important role in the progression of a significant subset of breast cancers.

We have made progress toward addressing the hypothesis that disrupting certain ERR α -co-activator interactions with small molecular weight compounds will decrease the transcriptional activation of ERR α target genes. One, we have identified a truncated form of ERR α that is constitutively active in cervical (HeLa) and mammary (BT-474, MCF-7) carcinoma cell lines. Two, we have identified in transient transfection experiments that the p160-family member, glucocorticoid receptor interacting protein 1 (GRIP1/SRC-2/TIF2/NcoA-2), is a specific co-activator of truncated ERR α -dependent, ERE-regulated transcription in MCF-7 cells. Three, we have prepared and characterized a panel of murine monoclonal antibodies to ERR α and GRIP1 that have proven useful in many applications. Four, we have developed a first-generation, cell-based, high-throughput screen (HTS) to search for potential inhibitors of GRIP1-mediated activation of truncated ERR α . This assay uses MCF-7 cells co-transfected with DNA vectors carrying truncated ERR α , GRIP1, and a firefly luciferase reporter gene containing EREs in its promoter. Luciferase activity is decreased in cells treated with a small molecule (a "hit") that interferes with the ERR α -GRIP1 interaction. Five, we have carried out an initial HTS of 9,400 compounds and have identified 34 hits that inhibit luciferase expression by greater than 75% and are not generally toxic to cells. Last, we are optimizing secondary assays to help us identify false-positive hits: a cell-based HTS in which the luciferase gene is driven by a promoter unaffected by ERR α and GRIP1 activity and an HTS using the protein fragment complementation assay (PCA), which will measure GRIP1-truncated ERR α protein-protein interactions in a cellular environment. We will continue in our efforts to identify novel compounds that interfere with the binding of ERR α to GRIP1. With proper pharmaceutical improvements, such an inhibitory compound could lead to the development of a novel breast cancer therapeutic. We hope that the compounds we identify will help patients with aggressive breast cancers that are hormone receptor-negative and/or resistant to currently available therapies.

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P15-3: EFFECTS OF SYNTHETIC DISINTEGRINS ON BREAST CANCER IN MICE

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Integrins are plasma membrane receptors. Many integrins bind peptide ligands containing the Arg-Gly-Asp sequence, and mediate cell interactions with matrix and other proteins. It has long been known that engagement or clustering of integrins and other receptors with multivalent reagents, such as antibodies, can induce different signaling responses than interaction with monovalent ligands. We hypothesized that divalent agents, depending on size, would affect breast cancer growth, tumor vascular permeability, or response to chemotherapy. These "disintegrins" were synthesized by conjugating cysteine-containing peptides, H2N-Gly-Arg-Gly-Asp-Ser-Pro-Gly-Gly-Cys-COOH (G) or a control H2N-Gly-Arg-Ala-Asp-Ser-Pro-Gly-Gly-Cys-COOH (A), which does not interact with integrins, to maleimide groups at the ends of linear 3.4, 6, or 10 kDa polyethylene glycol polymers (PEG). This created G3, G6, and G10, and the analogous A conjugates. Conjugation was confirmed by labeling with fluorescein isothiocyanate (reacts with amino termini), electrophoresis, and fluorescence imaging of gels. The agents were screened for activity in cultures of murine endothelial cells (EC). G10 inhibited EC adhesion to plastic and fibronectin in vitro. All other agents were inactive. EC were cultured in a transwell apparatus, and the effect of the agents on permeability of monolayers to fluorescein dextran was tested. As expected, vascular permeability/endothelial growth factor increased permeability of monolayers to 250 kDa dextran. However, none of the G conjugates increased permeability in vitro. The effects of the conjugates were tested on tumors of EMT6 adenocarcinoma cells transplanted in the 4th mammary fat pad of female BALB/c mice. Based on activity in the in vitro cell adhesion assay, the effect of G10 and A10 on tumor vascular permeability was probed using the albumin-binding dye, Evan's Blue, ip. Tumors were injected directly with PEG conjugates. One hour later mice were treated with dye, and tumors were harvested after another hour. Dye was extracted and measured by absorbance at 610 nm. G10 increased dye content in comparison with A10, indicating that it enhanced vascular permeability in vivo. To determine the effect of synthetic disintegrins on chemotherapy, tumors were injected with saline or PEG conjugate, and after 1 h mice were treated with 100 mg cyclophosphamide/kg, ip. Tumor size was non-invasively monitored. G10 combined with cyclophosphamide significantly reduced tumor growth compared with saline or cyclophosphamide alone or combined with A10. G or A conjugated to 3.4 and 6 kDa PEG were all ineffective. In the absence of cyclophosphamide, intratumoral G10 inhibited growth compared to A10. The results indicate that G10 inhibited EC adhesion, but not permeability, in vitro. In vivo, G10 reduced tumor growth, increased tumor vascular permeability, and, more significantly, potentiated the anti-tumor effect of cyclophosphamide. Thus, conjugation of an integrin-binding peptide to ends of a sufficiently large PEG produced an agent with anti-tumor activity.

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P15-4: SMALL MOLECULE INHIBITORS OF CADHERIN-11Stephen Byers,¹ Siva Dakshanamurthy,² and Milton Brown³¹Lombardi Comprehensive Cancer Center, ²Georgetown University Medical Center, and ³Georgetown University

Loss of expression or function of the epithelial cell-cell adhesion molecule E-cadherin is associated with poorly differentiated breast cancers. However, several studies have concluded that E-cadherin loss may be necessary but is not sufficient to promote invasion and metastasis. In fact, preliminary results point to increased expression of a mesenchymal/osteoblast cell-adhesion molecule, cadherin-11 (OB-cadherin), as a far better predictor of the invasive and metastatic phenotype in human breast cancers. In this work the laboratories of a cancer biologist and a medicinal chemist have combined to develop, refine, and test small molecule inhibitors of cadherin-11 function. We show that knockdown of cadherin-11 reduces growth and invasion in vitro and completely prevents tumor formation in aggressive breast cancer cells. In addition, cadherin-11 is the only known regulator of the lymphangiogenic factor VEGF-D. Taken together these data indicate that expression of cadherin-11 by breast cancer cells could potentially facilitate two characteristic aspects of breast carcinogenesis. First, increased cadherin-11 expression could lead to increased expression of VEGF-D leading to lymphangiogenesis or angiogenesis. Second, cadherin-11 could promote a migratory invasive phenotype leading to local invasion and lymph node metastases. The crystal structure of cadherin-11 was recently solved and revealed two potential sites for interference of cell-cell adhesion. We used this structure as a basis for molecular simulations and the production of pharmacophores designed to block one or both of two adjacent regions we anticipate are necessary for cadherin-11 function in cell-cell adhesion. We used our newly developed Common Reference Binding Mode (CRBM) strategy to select cadherin-11 inhibitors. The CRBM strategy requires that for each ligand docking simulation, the most common repetitive binding conformation be selected as a common reference binding mode. To account for protein flexibility, we use molecular dynamics (MD) simulations in conjunction with CRBM. Several potential lead compounds active in the 100 nM range have been identified on the basis of their ability to inhibit invasion in vitro of cadherin-11-expressing cells. These compounds are not cytotoxic and fail to block invasion of cells that do not express cadherin-11. These data clearly identify cadherin-11 as a potential therapeutic target in breast cancer and describe for the first time a strategy for the design of small molecule cadherin-11 inhibitors.

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P15-5: PROTEASE-MEDIATED NEAR-INFRARED FLUORESCENCE TUMOR IMAGING AND THERAPY

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A dual optical imaging and therapeutic agent was developed for tumor treatment. A fluorescent photosensitizer was assembled such that the fluorescence and phototoxicity are significantly quenched in their native state. The fluorescence and phototoxicity of the probe can be activated by the target proteases, thereby enabling near-infrared fluorescence imaging and selective treatment of tumors. To a poly-L-lysine/polyethylene glycol graft copolymer (PGC), multiple chlorin e6 (Ce6) photosensitizers were attached to induce self-quenching. Poly-L-lysine was selected because it is a substrate for several tumor-associated proteases. After optimization, the preparation with 15 Ce6 molecules per PGC molecule (L-SR15) showed about 86% fluorescence quenching comparing with free Ce6 and 4.0 times increase in fluorescence intensity after treating with a model protease, cathepsin B. Singlet oxygen generation (SOG) of L-SR15 was suppressed down to 13% of free Ce6 at equal molar concentration, and it was increased 6 times by cathepsin B treatment. When the L-polylysine was replaced by D-polylysine with similar substitution ratio (D-SR16), the same fluorescence quenching effect but no noticeable fluorescence activation or SOG was observed after cathepsin B treatment. In mice, the implanted HT-1080 tumors (a model cancer for cathepsin B activity) were clearly visualized by fluorescence molecular tomography 24 hours after intravenous injection of photosensitizers. Stronger fluorescence signal was observed from the tumor sections of L-SR15 injected group, whereas tumor sections from D-SR16 and Ce6 injected groups showed little or no fluorescence signal. When tumors were treated with light 24 hours post-injection, tumor reduction was only seen on the group that received L-SR15 plus light illumination, showing inhibition of tumor growth by over 50% after a single low dose (0.1mg/kg) of PDT.

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P15-6: DECONSTRUCTING NUCLEOTIDE BINDING ACTIVITY OF THE Mdm2 RING DOMAINCarol Prives, Christina Priest, Masha Poyurovsky, and Brent Stockwell
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The RING domain of Mdm2 contains a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins. As has been previously shown, Mdm2 preferentially binds adenine base nucleotides, and such binding leads to a conformational change in the Mdm2 C-terminus. (Poyurovsky et al. *Mol. Cell.* 12: 875-87, 2003). Nucleotide binding-defective Mdm2 mutants are impaired in p14^{ARF}-independent nucleolar localization, both in vivo and in vitro, and ATP-bound Mdm2 is preferentially localized to the nucleolus.

Here we present further biochemical analysis of the nucleotide-Mdm2 interaction. We confirmed the original ATP binding and specificity results using Isothermal Titration Calorimetry (ITC). Further investigation of the interaction using a series of ATP derivatives identified 2' and 3' hydroxyls of the ribose as well as the C6 amino group of the adenine base moiety as being essential for the interaction. These results further support our previous data on ATP specificity, as the C6 amino group is a unique feature of adenine. We have also shown that MdmX and Mdm2 family member with high sequence homology bind adenine nucleotides with similar affinity and specificity as Mdm2, suggesting that residues involved in nucleotide binding may be conserved between the two proteins.

To further elucidate the structural features of Mdm2 necessary for ATP interaction and to identify the portion of the Mdm2 C-terminus that is responsible for ATP binding, we have created a series of deletion and substitution mutations in residues within the Mdm2 RING domain. We assayed the resulting mutant proteins for nucleotide binding, nucleotide specificity, and E3 ligase activity. Using these assays, we have determined that the extreme 7 C-terminal residues of Mdm2, which are indispensable for Mdm2's E3 ligase activity, are not involved in nucleotide binding. Furthermore, we have mapped the specific adenine nucleotide binding region of Mdm2 to residues 429-484, encompassing the minimal RING domain. Our results highlight an intriguing separability between nucleotide binding and E3 functions of the Mdm2 RING domain, indicating that this domain may be involved in several unrelated biochemical processes.

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P15-7: THE β III ISOTYPE OF TUBULIN: ITS ROLE IN BREAST CANCER CELLS AND AS A POTENTIAL CHEMOTHERAPEUTIC TARGETRichard Luduena,¹ Tamas Bakos,² Asok Banerjee,¹ Starlette J. Y. Dossou,³ Richard Hallworth,³ Torin Huzil,⁴ Elzbieta Izbicka,⁵ Heather Jensen-Smith,³ Patrick Joe,¹ Corey Levenson,² Jonathan Mane,⁴ Veena Prasad,¹ Jack A. Tuszynski,⁶ Alexander Weis,² and I-Tien Yeh¹¹University of Texas Health Science Center at San Antonio, ²Oncovista, Inc.,³Creighton University, ⁴University of Alberta, ⁵Cancer Therapy and Research Center, and ⁶Cross Cancer Institute (CCI)

Background and Objectives: The microtubule subunit protein tubulin is a major target of antitumor drugs such as the taxanes. Tubulin is a heterodimer of two subunits designated α and β , each of which consists of multiple isoforms of different amino acid sequences. Of all the β isoforms, β III is the most interesting because it is overexpressed in many cancers, including breast cancer, and its distribution in normal tissues is limited. β III differs from most of the other tubulin isoforms in lacking a highly reactive cysteine at position 239 and in having a cysteine cluster at positions 124–129. We hypothesize that β III's function may be to protect cells against reactive oxygen species (ROS) and free radicals (FR). Because of β III's distribution, it could be an excellent target for antitumor drugs. Our objectives are (1) to determine the function of β III in breast cancer, and (2) to design and test drugs specific for β III.

Results: *Objective 1.* MCF-7 breast cancer cells treated with the oxidizing reagent diamide overexpress β III. Surveying a panel of excisions of human breast cancers, we have found a positive correlation between the aggressiveness of the cancer and the level of β III in the tumor. We purified the α II, α III, and α IV tubulin dimers from bovine brain and measured their ability to polymerize into microtubules in the presence of the FR superoxide. The ability of α II and α IV to polymerize was significantly inhibited by superoxide, but α III polymerized normally. Unfractionated tubulin, consisting of 25% α III, 58% α II and 14% α IV, was also normal, implying that α III exerts a protective function on microtubules.

Objective 2. We have done homology modeling on the human β -tubulin isoforms, focusing on the colchicine-binding site, with our goal to design drugs specific for the β III isotype of tubulin. We have designed and synthesized 24 such derivatives and

tested them in silico as well as in vitro against purified α II and α III isotypes and in vivo against cancer cells. Some of the drugs appear effective against cancer cells, less so against normal cells, and there is a correlation between predicted action in silico and actual behavior in vivo.

Conclusions: The β III isotype may play a role in protecting breast cancer cells against FR and ROS, which become particularly prominent in more aggressive cancers. The drugs we have designed to be specific for β III also appear to be effective against cancer cell lines and may be promising drugs for further development.

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P15-8: NF- κ B ACTIVITY AND METASTATIC POTENTIAL OF BREAST CANCER CELLS ARE INHIBITED BY THE NATURAL PRODUCT RESVERATROL AND BY SYNTHETIC ANALOGS

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Choice of adjuvant therapy for breast cancer largely depends on estrogen receptor (ER) status of the tumor and pathologic staging. Less aggressive ER-positive tumors are generally responsive to anti-estrogen therapies, while more aggressive ER-negative tumors typically require more forceful approaches such as combined radiation and chemotherapies. With time, advanced-stage tumors often become refractory to chemotherapeutic regimens and become metastatic due to activation of pro-survival and pro-metastatic signals. These signals are largely dependent on sustained activation of the nuclear factor κ B (NF- κ B) signal transduction pathway. Natural products such as the polyphenol resveratrol inhibit activation of the NF- κ B pathway in some cancer cells. It is often assumed that this activity of resveratrol and other polyphenols is dependent on their anti-oxidant properties. In the present study, resveratrol and synthetic analogs of resveratrol were shown to inhibit NF- κ B activity in MDA-MB-231 cells, which constitutively express NF- κ B. This determination involved transient transfection of these cells with an NF- κ B-dependent GFP construct and measurement of the inhibitory properties of resveratrol and analogs by flow cytometry. The anti-metastatic activities of resveratrol and analogs were determined using the Matrigel invasion assay. Resveratrol and analogs effectively inhibited MDA-MB-231 cell invasion, consistent with their abilities to inhibit activation of NF- κ B. Analogs devoid of anti-oxidant activity were effective, indicating that anti-oxidant activity is not essential.

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P15-9: VALIDATION OF SMALL MOLECULES DISRUPTING HEC1-NEK2 INTERACTION FOR BREAST CANCER TREATMENT

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Hec1 (highly expressed in cancer 1), which is located on the kinetochores and centrosomes, plays a critical role in chromosome segregation. The expression of Hec1 is highly elevated in most breast cancer cells. Inactivation of Hec1 leads to mitotic catastrophe due to abnormal chromosome segregation. It appears that phosphorylation of Hec1 by a serine/threonine kinase Nek2 is essential for its function. Since Hec1 is highly expressed in rapidly dividing cancer cells and inactivation of Hec1 results in cell death, we hypothesize that disruption of the interaction between Hec1 and its critical partner Nek2 may result in killing the breast cancer cells. Therefore, three small molecules, INH1, INH2, and INH3, were identified to disrupt the interaction between Hec1 and Nek2 through a reversed yeast two-hybrid screening. To elucidate the binding of lead compounds with its target protein, we have utilized the Biacore system and Affi-gel10 beads assay, which both demonstrated that INH compounds bind to Hec1, not Nek2. To establish the binding model by x-ray crystallography, we first purified the full-length Hec1 and coiled-coiled domain Hec1 and then tested over 2,000 buffer conditions for crystal formation. So far, several promising crystal leads have been obtained for diffraction. In addition, we also synthesized 24 INH analogues, explored their biological activities in killing breast cancer cells, and found that several analogues exhibited the stronger bioactivities than the leading compounds INH1-2. According to the results of bioactivities, we also explored the structure-activity relationship (SAR) of these analogues. Further optimization of these leading compounds and optimization of crystal formation conditions are currently in progress.

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P15-10: TARGETED DOWNREGULATION OF c-Myc THROUGH DISRUPTION OF THE TRANSCRIPTION FACTOR-G-QUADRU- PLEX COMPLEX

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Overexpression of the c-Myc oncogene plays a role in carcinogenesis in a variety of cancers, including cervical, breast, and colon, due to dysregulation of cellular growth and apoptosis. Studies have shown that up to 90% of c-Myc promoter activity is tightly controlled by the formation of a G-quadruplex secondary structure in the NHE III₁ region of this promoter. The formation of the secondary structure silences gene transcription and, subsequently, translation. Using high-throughput screening techniques, such as fluorescence resonance energy transfer (FRET), a group of compounds was identified that stabilize the c-Myc G-quadruplex structure. One compound (GSA-0612) decreased fluorescence by 50%, indicating a stabilization of the G-quadruplex structure. Further cell-free studies also confirm the FRET findings. Circular Dichroism demonstrates a dose-dependent stabilization of the G-quadruplex, with a shift in melting temperature of up to 7°C. By Taq polymerase stop assay, GSA-0612 (10 μ M) increased stop product by 30%. Using a breast cancer cell line with high c-Myc expression (MCF-7), the compound of interest was shown to decrease c-Myc gene expression by up to 97% at 48 h. This decrease translated into a downregulation of protein expression of c-Myc and downstream effectors, such as Cyclin D. Together, these data demonstrate a proof of principle by characterizing the activity of small molecular agents with a novel mechanism of transcriptional regulation. These agents, and potential analogs derived from them, hold great promise for the development of a unique method of treatment of breast cancer.

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P15-11: BIPHENYL C-CYCLOPROPYLALKYLAMIDES AS NEW SCAFFOLDS FOR TARGETING ESTROGEN RECEPTOR β

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Estrogens play a major role in the growth and development of a wide range of normal and cancerous tissues. Originally, it was thought there was only one estrogen receptor (ER) responsible for the biological effect of 17 β -estradiol (E2), estrogen receptor α (ER α). However, a second ER was discovered recently, estrogen receptor β (ER β). There are a few differences in the structure of the receptors and their tissue localizations are distinct. There is much data demonstrating the benefits of the selectively targeting the ER β . In breast cancer, E2's proliferating actions mediated through ER α have been demonstrated to be opposed by ER β . Development of a selective ER β agonist could also serve a role as treatment in central nervous system disorders and prostate cancer. Several subtype-selective compounds have been identified. The plant-derived compound genistein has significantly higher affinity for ER β . Additionally biphenyl-containing compounds have recently been investigated for ER β selectivity. Previously, through screening a library of homoallylic amides, allylic amides, and C-cyclopropylalkylamides, Janjic et al. identified a new structural scaffold for antiestrogens. The lead compound from this screening was used to design and synthesize a second-generation library, which was screened for ER α binding. In the present analysis, we screened this second-generation library for the ability to compete with a fluorescently labeled E2 derivative (ES2) for binding to human recombinant ER β using a fluorescence polarization (FP) assay. The library's efficiency of displacement at the ER β was significantly lower than that for ER α . In fact, only one compound demonstrated concentration-dependent displacement with close to 50% displacement at the highest concentration tested, 5 μ M. This compound did not satisfy the criteria for being a hit on ER α ; therefore, it was chosen as the lead structure for design of compounds with improved ER β selectivity. Because none of the compounds reached 50% displacement on ER β , the selection criteria were modified to include structural features demonstrated to previously promote ER β binding. Six analogues were then synthesized in an attempt to more selectively target ER β and improve in vitro and cellular potency. The compounds were first evaluated for their ability to compete with ES2 for binding to both ERs using the FP assay. Two compounds demonstrated selectivity for ER β . The compounds were also evaluated for their ability to inhibit E2-induced proliferation of human breast cancer cells using MCF-7 ER+ breast cancer cells. The two compounds demonstrated to be selective for ER β also had low GI50 values. None of the test compounds demonstrated significant growth inhibition of the ER negative human breast cancer cell line MDA-MB231. We have begun an investigation into modifying the biphenyl C-cyclopropylalkylamide pharmacophore to selectively target ER β . This allows for the development of new chemical tools to assist in studying the complex biology of the ERs and their interplay.

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P15-12: DEVELOPMENT OF A BREAST CANCER SELECTIVE THERAPEUTIC AGENT

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Background: The DNA synthetic machinery, (i.e., the DNA synthesome), of human breast cancer cells is significantly more error-prone than its normal breast cell counterpart. The increased frequency of mutations generated during DNA replication also correlates with the appearance of a unique isoform of a component of the synthesome (proliferating cell nuclear antigen [PCNA]). The isoelectric point of the breast cancer associated PCNA (caPCNA isoform) dramatically differs from that of its normal cell counterpart (nmPCNA) and results from post-translational modification and not by mutation or alternate splicing. Protein sequence analysis of caPCNA identified a short peptide segment (domain) of the protein that was hidden in normal cells but exposed in cancer cells. The cancer-specific nature of this domain was validated by IHC analysis of breast cancer biopsy tissue, employing antibody made to this peptide (>99% sensitivity and >93% specificity for breast cancer) and showing the peptide's unique ability to sensitize breast cancer cells to DNA damaging drugs (i.e., Doxorubicin [Dox]). We then hypothesized that this cancer-specific domain within the breast cancer cell caPCNA could have therapeutic potential and could be exploited for identifying breast cancer selective chemotherapeutics.

Objectives and Methodology: We are currently: (1) evaluating the caPCNA peptide for its ability to interfere with the binding of cancer cell DNA repair proteins to full-length caPCNA protein; thereby affecting their function; using (a) biochemical assessment of the caPCNA peptide's ability to block the binding of caPCNA to proteins involved in DNA repair and (b) evaluation of the peptide's ability, +/- Dox, to directly affect different DNA repair functions; (2) defining the structural elements of the caPCNA peptide that are critical to its function with Dox by: substituting individual amino acids (aa) within the cancer-specific caPCNA domain with Alanine (Ala) and determining which of these (aa) are absolutely required for: (a) the observed cooperative killing of cancer cells with caPCNA peptide + Dox, (b) the specific binding of known caPCNA protein partners, and (c) the peptide's effect on DNA repair functions; (3) establishing the usefulness of the cancer-associated domain of caPCNA as a cancer-specific therapeutic target. This is being accomplished by: (a) assaying the interaction of chemical library components with the cancer-specific domain of caPCNA to identify chemical agents that target this sequence. Once identified, these agents will then be used to assess their capacity to specifically inhibit (i) tumor cell proliferation in culture and (ii) tumor growth in an animal model.

Results and Conclusions: Since funding commenced in November 2007, we have identified an N-terminal amino acid sequence that enables the caPCNA peptide to be rapidly taken up by cells, and we have shown that as little as 10 μ M of the peptide will rapidly kill only cancer cells. The peptide sensitizes these cells to Dox; widening the therapeutic window by reducing the IC₅₀, while increasing its effectiveness. Alanine scanning mutant peptides have been ordered and 2 chemical libraries: (i) a 66,000 compound discovery library and (ii) a 200,000 compound peptide-like chemical library have been ordered. We have also received IACUC approval for use of our mouse tumor model.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0707 and National Cancer Institute.

P15-13: A NOVEL APPROACH TO THE DEVELOPMENT OF SMALL MOLECULE INHIBITORS OF Cdc25 PHOSPHATASE

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Cdc25 phosphatases are key regulatory proteins of the eukaryotic cell cycle. Their overexpression is associated with numerous cancers making them compelling targets for the design and development of anti-cancer agents. Consequently, these proteins have been the subject of extensive efforts aimed at developing small molecule active site inhibitors, but these have proven largely unsuccessful. Recently, a remote hotspot of Cdc25 was discovered that is critical to substrate binding. Using computational methods, we have probed this region with a library of small molecules and identified several lead structures as potential ligands of an associated surface pocket. We report here the total synthesis of a focused library of related compounds and its initial in vitro screening for potential inhibitors of Cdc25 phosphatase.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0514.

P15-14: UNDERSTANDING AND TARGETING MYC ONCOPROTEIN FUNCTION IN BREAST CANCER

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Activation of the MYC oncogene has been detected in early- and late-stage disease and is often associated with poor prognosis, suggesting a more aggressive, and/or Myc-targeted, treatment strategy may be appropriate with cancers that show evidence of this potent oncogenic lesion. The Myc oncoprotein is a key node downstream of multiple signaling cascades, a regulator of gene transcription that drives the neoplastic process in approximately 50% of all breast cancers. Deregulated expression of Myc is due to both amplification of the MYC locus at a frequency of 25% (range, 4%–52%), as well as overexpression in the absence of elevated copy number (~25%). In mouse models of cancer, Myc deregulation can initiate mammary carcinogenesis and inactivation of Myc expression has been shown to dramatically reduce the tumor mass and even eradicate disease. Evidence shows even a modest suppression of Myc expression can markedly block tumor cell growth. Thus, identifying the subset of target genes regulated by Myc that are essential for transformation and targeting this function of Myc with novel anti-cancer therapeutics would mark a key advance.

To fill this gap we will exploit evidence that Myc partners with another molecule called TRRAP to regulate genes required for tumorigenesis. We have recently identified the regions of TRRAP that interact with Myc and we aim to exploit this knowledge to understand the mechanism of Myc action as an oncogene in breast cancer. To delineate the genetic program regulated by Myc and TRRAP in transformation, we have recently characterized a mammary cell system that is transformed in a Myc-dependent manner. With this system, we are using chromatin immunoprecipitation (ChIP) combined with microarrays (chip) to capture the gene regulatory regions bound by Myc and TRRAP in breast cancer to drive transformation. Preliminary evidence suggests that the Myc-induced and Myc-suppressed genes identified will in turn function as oncogenes and tumor suppressors, respectively. This research will identify functionally important targets for further therapeutic development and validate the importance of TRRAP in Myc-directed transformation. We are also advancing this research to inhibit Myc function and kill breast cancer cells by targeting Myc:TRRAP interaction with small molecules. We are presently developing novel high-content, live cell screens to identify lead compounds. The results of both our molecular characterization of Myc and TRRAP function in transformation and the development of specific inhibitors targeting this oncogenic interaction will be presented and discussed.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0333.

P15-15: HIGH-CONTENT FRET-FLIM SCREENING FOR INHIBITORS OF ONCOGENIC TRANSCRIPTION BY c-MYC IN BREAST CANCER

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Breast cancer has directly or indirectly affected the lives of all North Americans. Cancer results when DNA in a single cell accumulates multiple mutations that trigger the cell to grow in an uncontrolled manner. To develop drugs effective against a wide spectrum of cancers, we are focusing our research efforts on a particularly potent cell growth gene called Myc, which is often misregulated in breast cancer.

There is evidence that Myc partners with another molecule called TRRAP to regulate genes required for transformation. Our hypothesis is that blocking the interaction of Myc with TRRAP will selectively kill breast cancer cells. Traditional drug screening approaches to identify compounds that will break apart two interacting proteins have rarely been successful. Our innovative approach to solving this problem is to screen compounds in live breast cancer cells for the ability to prevent Myc and TRRAP from binding to each other in the first place. Since the cell is constantly replacing Myc, a compound that prevents it from binding TRRAP should kill even established tumors. Importantly, we previously mapped the places on Myc and TRRAP that bind them together and showed they don't involve the parts of the protein involved in other important functions. Therefore, the compounds we find that prevent Myc-TRRAP binding should not be toxic to normal cells.

We have established a new assay in which Myc and TRRAP are expressed as fusion proteins to Cerulean and Citrine fluorescence proteins, respectively. The interaction between the two proteins results in fluorescence resonance energy transfer (FRET) between the Cerulean and Citrine fluorescence proteins. As a result of the FRET be-

tween Cerulean and Citrine, the fluorescence lifetime of Cerulean decreases from 2.7 to 2.0 ns. Therefore we can detect FRET by fluorescence lifetime imaging (FLIM). A novel robotic microscope that can automatically perform FLIM and thereby detect Myc-TRRAP binding quickly and accurately has been assembled and tested. Using this microscope we can test individually the effect of off-patent drugs and drug-like molecules in live breast cancer cells. This will let us identify compounds that are not only effective but that get into breast cancer cells and are not toxic to normal cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0333.

P15-16: STRUCTURE-ACTIVITY RELATIONSHIP ANALYSIS OF RAT MAMMARY CARCINOGENS

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Structure-activity relationship (SAR) models are powerful tools to investigate the mechanisms of action of chemical carcinogens and to predict the potential carcinogenicity of untested compounds. We describe here the application of the cat-SAR (categorical-SAR) program to two learning sets of rat mammary carcinogens. One set of models developed was based on a comparison of rat mammary carcinogens to rat noncarcinogens (MC-NC), and the second compared rat mammary carcinogens to rat nonmammary carcinogens (MC-NMC). The best rat MC-NC model achieved a concordance between experimental and predicted values of 82%, a sensitivity of 78%, and a specificity of 87%. The best rat MC-NMC model achieved a concordance of 78% with a sensitivity of 82% and a specificity of 73%. The MC-NMC model was based on a learning set that contained carcinogens in both the active (i.e., mammary carcinogens) and inactive (i.e., carcinogens to sites other than the mammary gland) categories and was able to distinguish between these different types of carcinogens (i.e., tissue specific) not simply between carcinogens and noncarcinogens. Based on a structural comparison between this model and one for *Salmonella* mutagens, there was, as expected, a significant relationship between the two phenomena since a high proportion of breast carcinogens are *Salmonella* mutagens. However, when analyzing the specific structural features derived from the MC-NMC learning set, a dichotomy was observed between fragments associated with mammary carcinogenesis and mutagenicity and others that were associated with estrogenic activity. Overall, these findings suggest that the MC-NMC model is able to identify structural attributes to address the question of "why do some carcinogens cause breast cancer," which is a different question than "why do some chemicals cause cancer."

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P15-17: BIOACTIVE ANTIESTROGEN CONJUGATES SHOW UNIQUE MECHANISM OF ACTION

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Background and Objectives: Drugs targeting estrogen action are used in the treatment of breast cancer, but the major problem with these therapies is that the effects of the drug are not limited to the target tissue. A possible explanation for these tissue-dependent effects is the existence of multiple estrogen receptors that can signal from different parts of the cell such as the nucleus, cytoplasm, mitochondria and plasma membrane. The purpose of this research has been to synthesize new compounds in which the antiestrogen drug is attached to various molecules that could dictate localization to certain subcellular locations to determine what role those receptors play in breast cancer proliferation and the development of antiestrogen resistance.

Methodologies and Results-to-Date: Initial work was started with various conjugates to 4-hydroxytamoxifen. A small structure-activity relationship study identified a suitable substitution site off of the basic side chain of 4-hydroxytamoxifen. Conjugates were synthesized that attached multiple 4-hydroxytamoxifen analogs to a 25 kDa poly(acrylic acid) polymer. The polymer-4-hydroxytamoxifen conjugate bound to the estrogen receptor with high affinity in vitro and was then found to be a potent antagonist of estrogen receptor-mediated transcription in breast cancer cells, indicating efficient cellular uptake and intracellular activity. Tagging the polymer conjugate with a fluorescent tag showed very high levels of uptake into cells and a uniform cytoplasmic distribution. As a control, the fluorophore was directly attached to the 4-hydroxytamoxifen analog and a similar cellular distribution was seen. Simultaneous monitoring of estrogen receptor localization suggested that the receptor was shuttled entirely out of nucleus and was not degraded, suggesting a novel mechanism of estrogen receptor antagonism. The conjugates also stimulate rapid estrogen signaling responses in different breast cancer cell lines. Other conjugates have been synthesized based on estradiol analogs and are currently being investigated for their similarities to the 4-hydroxytamoxifen analog conjugates.

Conclusions: While we have achieved localization to either the nucleus or cytoplasm, localization to the plasma membrane still proves elusive. Even with this setback, there are still three major findings from this work that have potential clinical impact. The first finding is a new possible mechanism of estrogen receptor antagonism that is different from the mechanisms of tamoxifen or fulvestrant. This could add another option for ER-positive breast cancer that could overcome certain anti-estrogen resistance mechanisms. The second major finding is that the polymer-conjugate is as effective at inhibiting breast cancer cell proliferation as 4-hydroxytamoxifen. This is important because the function of this polymer conjugate in other tissues such as the uterus and uptake across the blood-brain barrier could be very different than 4-hydroxytamoxifen. This could result in a drug with much better side effect profile than tamoxifen. The third major finding is that the conjugation of the 4-hydroxytamoxifen analog results in very efficient uptake of relatively cell-impermeable scaffolds such as the polymer we used. This could be manipulated to allow for the uptake of therapeutics with low cell permeability such as siRNA or certain chemotherapy drugs.

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P15-18: SELECTIVE KILLING AND IN VIVO IMAGING OF BREAST CANCER CELLS WITH SHORT SYNTHETIC DNA APTAMERS

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Background: The most promising future therapies for breast cancer will be the ones that will address the issue of targeted delivery. Specifically, delivery strategies are needed to recognize unique cell surface tumor markers on cancer cells, and the targeted surface determinants must represent useful gates or portals into cells. The choice of gates is crucial for breast cancer patients since the delivery of therapeutic cargoes to and into cells (cargoes being either drugs, peptides, proteins, RNA/DNA oligonucleotides, plasmids, or radionuclides) will determine if a treatment modality leads to a selective cure with minimal morbidity or toxicity issues.

Methods: The SELEX approach (systematic evolution of ligands by exponential enrichment) was used to identify short synthetic DNA oligonucleotides, termed aptamers, that recognize biosynthetically engineered GalNac-peptide markers known as Tn antigens.

Results: Tn antigens are unique tumor markers present on the surface of breast cancer cells (as in the case of T47D and MCF-7 cell lines) but not on normal mammary epithelium. We report the discovery of synthetic DNA aptamers that selectively bind with nanomolar affinities to Tn antigens and are rapidly internalized by such cells. These Tn antigen-specific aptamers have now been labeled with imaging agents and phototoxic drugs and were used to visualize and kill a breast cancer xenograft model established in nude mice.

Conclusions: The relevance of our findings to breast cancer patients stems from the fact that DNA oligonucleotides offer great potential as therapeutic agents, but they are charged molecules that are cell-impermeant agents. We have now derived DNA oligonucleotides (aptamers) specifically directed at tumor antigens that can serve as effective gates into breast and other epithelial cancer cells, an important finding that now suggests a powerful new role for DNA oligonucleotides as selective intracellular delivery vehicles for the diagnosis and treatment of breast cancer patients.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0392; Canadian Breast Cancer Research Alliance; and Canadian Cancer Society.

P15-19: MECHANISM OF SELECTIVE PROTECTION BY SILDENAFIL CITRATE AGAINST DOXORUBICIN-INDUCED APOPTOSIS IN NORMAL CARDIO-MYOCYTES AND SENSITIZATION TO DOXORUBICIN IN BREAST CANCER CELLS

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Virginia Commonwealth University

Doxorubicin (Adriamycin) is an anthracycline antibiotics frequently used in the treatment of acute leukemia and solid tumors such as advanced breast cancer, ovarian cancer, and lung cancer. However, the clinical efficacy of doxorubicin is frequently limited by the development of severe cardiomyopathy or congestive heart failure after acute or chronic treatment. Sildenafil, a selective phosphodiesterase 5 inhibitor, currently approved by FDA for the treatment of erectile dysfunction and pulmonary arterial hypertension, has shown promise as a possible pharmacologic cardioprotectant

against the cardiotoxicity of doxorubicin in animal studies. Furthermore, our preliminary studies have demonstrated that sildenafil fails to protect both MCF-7 and MDA-MB231 breast cancer cells from doxorubicin cytotoxicity; in addition, sildenafil was found to enhance doxorubicin sensitivity in MDA-MB231 cells, in part through the promotion of apoptosis.

In the current work, we exposed MCF-7 breast cancer cells to several frequently used chemotherapeutic agents with different mechanisms of action, including camptothecin, paclitaxol, and cisplatin as well as ionizing irradiation with or without sildenafil pretreatment. As with doxorubicin, sildenafil did not demonstrate cytoprotective effects against these drugs or radiation in either MDA-MB231 or MCF-7 cells. Sildenafil also showed the capacity to promote increased sensitivity to doxorubicin over a range of drug concentrations in MCF-7/caspase-3 cells (engineered to express caspase 3). As previous results demonstrating potentiation of sensitivity to doxorubicin by sildenafil were based on the MTT assay, clonogenic survival assays were performed to evaluate the response to sildenafil and doxorubicin in MCF-7, MCF-7/caspase 3 and MDA-MB231 cells. Sildenafil did not demonstrate protection and in fact moderately enhanced sensitivity to doxorubicin, supporting the results of our preliminary work.

Doxorubicin-induced cell death in cardiac myocytes is thought to occur through free radical generation while cell death in breast tumor cells is primarily through topoisomerase II-mediated double-strand breaks. We initiated studies of doxorubicin toxicity in an embryonic H9C2 cardiomyocyte cell line. The free radical scavenger, N-acetyl-cysteine, partially prevented hydrogen peroxide or doxorubicin-induced cell death. However, sildenafil did not show cytoprotection against doxorubicin based on trypan blue exclusion or the TUNEL assay as an indication of apoptosis.

A possible explanation for the differential effects of sildenafil cardioprotection relates to the use of primary cardiomyocytes in previous work and embryonic cardiac cells that can reproduce in the current studies. We are currently examining specific elements, such as the levels of phosphodiesterase-5, iNOS or eNOS and PKG expression, to determine the factors that could be involved in the putative cardioprotective effects of sildenafil.

The potential impact of this work relates to the possibility of protecting breast cancer patients from the cardiotoxicity of doxorubicin without interfering with the antitumor action of this or other chemotherapeutic agents.

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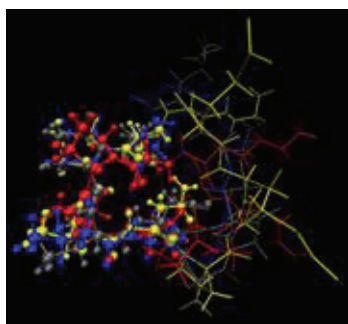
P15-20: COMPUTATIONAL DESIGN OF A SMALL PEPTIDE THAT INHIBITS BREAST CANCER

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Breast cancer is the most common cancer diagnosed in women and is the second leading cause of cancer death among women, closely following lung cancer. Many breast cancer drugs, like tamoxifen, have limited long-term efficacy and often have undesirable side effects, leading researchers to search for new breast cancer drugs.

Alpha-fetoprotein (AFP), an embryo-specific serum alpha-globulin glycoprotein, is synthesized by the fetal yolk sac and circulates through the serum of pregnant women. A growth-regulating hormone, AFP has the capacity to both stimulate and inhibit growth, although scientists remain uncertain of the exact pathways involved. In the last several decades, clinical researchers have discovered the anti-estrogenic cancer properties of AFP. A number of studies have since shown its effectiveness as a therapeutic agent to treat estrogen-dependent breast cancer, as well as its ability to prevent premalignant foci from developing into breast cancer.

Researchers at Albany Medical College performed studies on AFP, parsing it down to a smaller peptide chain of 34 amino acids that retained the same anti-estrogenic activity as the original molecule. Continuing research attempted to identify smaller subchains of the original AFP molecule that remained active, finding an 8-mer that they named P472-2. Antiestrogenic activity was then measured in a uterine mouse assay. The original AFP molecule, its 34 amino acid analogue, and P472-2 all showed comparable activity in prohibiting estradiol-induced growth in the uterus. P472-2 has been shown to inhibit estrogen-induced T47D breast cancer cells in culture as effectively as the original AFP molecule.



Overlay of active region of peptides

Information about the pharmacophore has been previously obtained by substituting amino acids in P472-2. Cyclic analogs nine amino acids long have been synthesized that also retain full activity. All efforts to create a peptide under eight residues resulted in the loss of anti-cancer activity. We have used Replica Exchange Molecular Dynamics techniques to model selected active analogs of the linear and cyclic peptides to explore their conformational space. Our results reveal that the peptide's critical region is a four amino acid sequence that has a turn conformation. We present here a new lead compound that shows anti-cancer activity as measured in T47D breast cancer cells in culture and in a uterine mouse assay.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0441 and National Science Foundation.

P15-21: DISCOVERY OF BREAST CANCER SERM MOLECULES: NOVEL USE OF FUNDAMENTAL QUANTUM MECHANICS

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The National Cancer Institute has identified a need to discover better selective estrogen receptor modulators (SERMs). It was our concept to discover SERM molecules through the quantum mechanical topology of the electron density. The best topological pharmacophore is the Laplacian of the density, as it determines where electronic charge is locally concentrated and depleted. The physical significance of this is that a SERM and an estrogen receptor must have complementary Laplacians for an effective bonding interaction to occur between them. Because the estrogen receptor is a large biological molecule, the calculation of its electron density by abinitio quantum mechanics presents a challenge. Our familiarity with the kernel energy method (KEM) made us aware of its usefulness in treating large biological molecules. The essence of the method is that a large biological molecule is broken into smaller pieces called kernels. The energy and density of the full molecule are obtained by certain summations over those of the kernels. We considered the extension of the KEM to a fourth order of interaction among the kernels that compose a biological molecule. The results of extending the KEM to fourth order are highly accurate.

An indication of the high accuracy obtainable by the KEM extended to a fourth order of interaction is shown in table 1. As the order of the interactions included go from single to double to triple to quadruple, ΔE the difference between the exact answer and the calculated KEM approximation goes toward zero.

Table 1. The Hartree Fock STO-3G Energy Calculated up to Fourth Order of KEM, for Leu¹-Zervamicin (Closed Form) 265 Atoms

Energy	Single	Double	Triple	Quadruple
EKEM in [au]	-5857.8663	-5851.5469	-5851.5686	-5851.5703
Exact in [au]	-5851.5703	-5851.5703	-5851.5703	-5851.5703
ΔE in [au]	6.2960	-0.0234	-0.0017	0.0000

Thus we now have a way to obtain accurate quantum electron densities for the estrogen receptor alone and in interaction with its SERM molecules. We have therefore paved the way to use the Laplacian of the electron density as a practical quantum topological pharmacophore. This suggests a fundamental new methodology to discover improved SERM drugs, prevent breast cancer, and promote general health, consonant with National Cancer Institute's goals.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0658.

P15-22: STRUCTURAL CHARACTERIZATION AND DETERMINANTS OF SPECIFICITY OF SINGLE-CHAIN ANTIBODY INHIBITORS OF MEMBRANE-TYPE SERINE PROTEASE 1

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There is an urgent need to understand how to design better therapeutic agents to aid in the understanding and treatment of breast cancer. Proteolytic enzymes have been implicated in all stages of cancer progression including primary tumor growth, invasion, migration, angiogenesis, and metastasis, but it has been difficult to develop effective inhibitors to these enzymes due to potency and specificity issues. Membrane-type serine protease 1 (MT-SP1/matriptase) is one of many cancer-associated serine proteases implicated in the tumorigenesis and metastasis of breast cancer. We have developed a number of potent and specific single-chain (scFv) antibody inhibitors to MT-SP1 and have characterized their mechanism of inhibition. Through kinetic experiments, site-directed mutagenesis experiments, and x-ray crystallography, we have the mechanism of inhibition of the most potent antibody-based inhibitors. The inhibitors have similar, but distinct mechanisms of inhibition that do not mimic

either biologically or pharmaceutically relevant protease inhibitors; they gain potency and specificity through interactions with the protease surface loops and inhibit by binding in the active site in a catalytically noncompetent manner. In contrast to most naturally occurring protease inhibitors, which have diverse structures but converge to a similar inhibitory archetype, antibody inhibitors provide an opportunity to develop divergent mechanisms of inhibition from a single scaffold. These results suggest that the development of specific antibody-based inhibitors against individual members of

closely related enzyme families is feasible and an effective way to develop tools to tease apart complex biological proteases. The results of this work are helping to rationally guide our implementation of protease inhibitors on antibody scaffolds to better understand the roles of proteases in breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0300.

EXPERIMENTAL THERAPEUTICS I

Poster Session P16

P16-1: DEVELOPMENT OF A CHEMOKINE (SDF-1/CXCL12) ENHANCED TRANSGENIC MOUSE METASTASIS MODEL FOR PRECLINICAL TESTING OF NOVEL ANTI-CANCER DRUGS

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Current treatment modalities are rarely curative following widespread metastasis in patients with breast cancer (BCa). Injection of established human BCa cell lines into mammary tissue of immunodeficient (ID) mice has provided considerable insight into tumor growth. However, most human BCa cell lines have limited spontaneous metastatic capacity in mice thus limiting progress in developing novel anti-metastatic therapy. Recent studies have demonstrated that signaling between the chemokine, stromal cell-derived factor (SDF-1; CXCL12) produced in target organs (bone, lymph nodes, lung, liver) and its cognate G-protein-coupled receptor, CXCR-4, highly expressed on human BCa cells, plays an important role in directing cancer cell homing to metastatic organs. Our hypothesis is that constitutive secretion of SDF-1 by target organs in ID mice is a limiting factor in spontaneous metastasis from human BCa xenografts. We propose that BCa metastases will be markedly enhanced in mice producing high levels of SDF-1. Our initial goal was to demonstrate that supraphysiologic levels of SDF-1 would enhance metastases of human BCa cells (1×10^6) injected orthotopically into the mammary fat pad of ID mice. Following orthotopic implantation, spontaneous MDA-MB-435 cancer metastases were observed in the lungs of ~50% of mice. Pathologic examination revealed metastases primarily in small lymphatic vessels of the lungs with subsequent invasion of lung parenchyma. In our initial plan, we proposed to inject adenoviral vector expressing SDF-1 (AdSDF1) intravenously into groups of mice that would then be sacrificed at intervals to quantify metastases. In our initial experiment designed to demonstrate the biologic activity of the SDF-1 virus vector, the vector was injected intravenously into NCr nu/nu mice at three different doses 2×10^8 , 1×10^9 , and 1×10^{10} plaque forming unit (pfu). As a control, Ad-Null (no transgene) virus was injected into mice at the same doses. The results indicated that mice injected with Ad-SDF-1 virus did not develop increased white blood cell counts or increased plasma levels of SDF-1 as compared to mice injected with AdNull virus. These results indicate that the Ad-SDF-1 virus that we were injecting was not producing the biologic effect anticipated. Hence, further viral injection experiments were halted. Plasma levels of SDF-1 (immunoassay) were increased in both Ad-Null and Ad-SDF-1-treated mice at the dose of 1×10^9 pfu as compared to control mice. These data suggest that the virus vector alone has an effect on production of SDF-1. To determine whether the SDF-1 effect might be delayed in these mice, a second dose of virus vector was injected into mice on day 5, and WBCs were counted on day 8; no significant increase in WBC counts were noted, confirming that the SDF-1 vector was nonfunctional. The second component of the grant was to develop transgenic ID mice engineered to produce human SDF-1 under the control of a human cytomegalovirus promoter/enhancer sequence and linked to a human growth hormone intronic and polyadenylation sequence. These mice would then be evaluated for spontaneous metastasis following orthotopic injection of human BCa cell lines previously characterized as producing varying levels of CXCR-4 (MDA-MB-361>MDA-MB-231>MCF-7>T-47D). Correlations would be sought between SDF-1 tissue levels and metastases.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0490.

P16-2: APTAMER TARGETING OF TUMOR CELLS

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Boston University, Boston Campus

The long-term goal of this research is to develop breast cancer imaging and drug delivery systems. This difficult goal has been sought by many researchers for >50 years because of the potential large impact on the detection and treatment of cancer. The research described here focuses on developing an emerging class of targeting molecules called aptamer against breast and other cancer cells. The ultimate goal is to add imageable labels to the aptamers to enable tumor cell detected and radionuclides for treatment in situ.

Aptamers are promising alternatives to antibodies ("antibody mimics") and are composed of single-stranded DNA that fold into stable 3-dimensional structures and bind to selected targets. The advantages of aptamers include their synthesis and selection in vitro from libraries of DNA sequences with 10^9 -fold greater complexity than an individual's antibody repertoire, small size, easy synthesis, storage, modification, stability, modulatable in vivo degradation rate, and general non-immunogenic and toxic with excellent biodistributions.

This research identified aptamers against carcinoembryonic antigen (CEA). CEA is present on ~50% of breast cancer tumor cell surfaces, as well as other adenocarcinomas, especially colon tumors. Aptamers were selected from chemical synthesized random single-stranded DNA libraries composed of 10^{14} different sequences using ten cycles of a two-step selection protocol. After each cycle, sequences bound to CEA were PCR amplified and used for the subsequent selection cycle. After 10 rounds of

selection, 100 randomly chosen oligonucleotides were cloned and sequenced. Sequence comparisons revealed several conserved motifs. In vitro testing of the individual sequences using a fluorescent polarization assay uncovered at least 2 sequences that have high affinities ($K_d = 10^{-9}$ M) and selectivity for CEA. This work is important because several aptamer sequences have been isolated that can be further developed as targeting reagents for both detection and treatment of breast cancers. Further, the research has established methods that can be used to isolated anti-breast cancer tumor aptamers against other epitopes (some invisible to antibodies) and even whole tumor cells efficiently.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4414 and Philip Morris External Research Program.

P16-3: THE PERVASIVE PRESENCE OF FLUCTUATING OXYGENATION IN TUMORS

Laura Isabel Cardenas-Navia,¹ Daniel Mace,¹ Rachel Richardson,¹ David Wilson,¹ Siqing Shan,¹ and Mark W. Dewhirst²

¹Duke University and ²Duke University Medical Center

Tumor hypoxia is a persistent obstacle for traditional therapies in solid tumors. Strategies for mitigating the effects of hypoxic tumor cells have been developed under the assumption that chronically hypoxic tumor cells were the central cause of treatment resistance. In this study we show that instabilities in tumor oxygenation are a prevalent characteristic of three tumor lines, and previous characterization of tumor hypoxia as diffusion-limited does not accurately portray the tumor microenvironment. Phosphorescence lifetime imaging was used to measure fluctuations in vascular pO_2 in rat fibrosarcomas, 9L gliomas, and R3230 mammary adenocarcinomas grown in dorsal skin-fold window chambers ($n=6$ for each tumor type) and imaged every 2.5 minutes for a duration of 60–90 minutes. O_2 delivery to tumors is constantly changing in all tumors, resulting in continuous reoxygenation events throughout the tumor. The fluctuations in oxygenation occur with a common periodicity within and between tumors, suggesting a common mechanism, but have tumor type dependent spatial patterns. The widespread presence of fluctuations in tumor oxygenation has broad-ranging implications for tumor progression, stress response, and signal transduction, which are known to be altered by oxygenation/reoxygenation events.

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P16-4: LOCOREGIONAL CHEMORADIOTHERAPY OF BREAST AND PROSTATE CANCERS USING ¹⁸⁸RE-CISPLATIN-HYDROGEL

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Objective: Site-specific delivery of anticancer agents or radiation is beneficial for solid tumors with poor blood supply or surgically unresectable tumors. ¹⁸⁸Re is a useful radiotherapeutic isotope producing gamma and beta rays. This study was aimed to determine the effectiveness of ¹⁸⁸Re-hydrogel for loco-regional therapy of breast and prostate tumors in animal models.

Methods: Rats or nude mice were inoculated with rat breast cancer cells or human prostate (PC-3) cancer cells. When tumor volume reached designated size, the rodents were injected with hydrogel alone, free ¹⁸⁸Re (1 mCi), ¹⁸⁸Re-hydrogel (1 mCi), and ¹⁸⁸Re-cisplatin-hydrogel (1 mCi, 250 µg). Tumor volume was measured as $L \times W \times H/2$ daily up to 25 days. The effectiveness of hydrogel was measured by tumor growth curve. Planar imaging was performed on days 0, 2, and 3 to monitor the localization of ¹⁸⁸Re within the hydrogel matrix. The rodents were sacrificed at various days, and blood chemistry (CBC, white blood cell counts, and serum enzymes) and histology were analyzed.

Results: The averaged days of tumor growth reaching 350 cubic mm with hydrogel, free ¹⁸⁸Re, ¹⁸⁸Re-hydrogel, and ¹⁸⁸Re-cisplatin-hydrogel were 6, 8, 13, and 21 days, respectively. ¹⁸⁸Re-cisplatin-hydrogel clearly demonstrated the best therapeutic effect in tumor volumes throughout the study. Imaging on day 0 showed minimal release of ¹⁸⁸Re from the hydrogel matrix as supported by ¹⁸⁸Re accumulation in the stomach and thyroid. Unbound ¹⁸⁸Re was eliminated through renal excretion and not present on any of the delayed images. Planar imaging showed ¹⁸⁸Re-hydrogel retained locally. There were no significant differences in BUN and creatinine levels among hydrogel and ¹⁸⁸Re-hydrogel and ¹⁸⁸Re-cisplatin-hydrogel groups.

Conclusion: Administration in situ hydrogel with high loading yields of ¹⁸⁸Re and anticancer drug matrix provides the best therapeutic effect without the leakage of ¹⁸⁸Re into surrounding tissues and with less drug-induced tissue toxicity.

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P16-5: A NOVEL COMBINATION OF THERMAL ABLATION AND HEAT-INDUCIBLE GENE THERAPY FOR BREAST CANCER TREATMENT

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High-intensity focused ultrasound (HIFU) can convert focused acoustic energy into heat and thus produce well-defined focal lesions in vivo. In recent years, HIFU has emerged as a promising treatment modality for breast cancers. However, the current HIFU therapy cannot eradicate 100% of the tumor volume or treat the metastatic cancer cells outside of the primary tumor site. We hypothesize that HIFU can not only destroy primary tumor tissue but also induce therapeutic effects in sublethally injured tumor cells. This investigation is to explore the potential synergistically combining HIFU thermal ablation and heat-induced gene therapy to improve the efficiency and overall quality of breast cancer treatment.

The HIFU exposure system was constructed by integrating a single element air-back annual HIFU transducer with a phased array ultrasound probe. Cells were transfected with heat-sensitive adeno-virus vectors. Inducible marker gene expression in vitro was evaluated in a thermal dosage-dependent manner by monitoring marker gene green fluorescent protein (GFP). An optimized HIFU exposure condition was thereafter resulted to guide an animal experiment on a tumor-bearing murine model. An in vivo bioluminescence imaging system was used to record luciferase distribution in the hind limbs of Balb/C mice subcutaneously inoculated with 4T1-hsp70B-Luc cancer cells. Furthermore, a 3D cell-embedded tissue mimicking phantom was developed. With this phantom model, the observed gene expression pattern was correlated with in situ delivered thermal dosage from HIFU exposure.

The HIFU system was applied to generate different exposure pattern and dosage while the acoustical and thermal fields were systematically determined. HIFU-induced gene expression was first investigated in the HeLa-hsp70B-GFP cell suspension, and the maximum HIFU-induced gene expression was achieved at 60° in 5s. The maximum luciferase gene expression in the tumor-bearing murine model was then achieved at a peak temperature of 65–75° within 10–20s. Thermal stress, in both in vitro and in vivo studies, was identified as the primary physical mechanism for HIFU-induced gene expression. In the 3D phantom study, GFP positive cells were primarily found within a circumferential region surrounding the primary site of lesion formation, which also coincides with the thermal necrosis boundary, indicating that gene expression was primarily induced in the sublethally injured cell population along the HIFU lesion boundary.

In conclusion, an image-guided computer-controlled experimental HIFU system was developed and characterized for both in vitro and in vivo studies. The efficiency of HIFU-induced gene expression under the control of hsp70B promoter was separately examined in cell suspension and tumor model. To elucidate the underlying physical mechanism, a 3D cell-embedded tissue mimicking phantom was developed with similar acoustic and thermal properties to breast tissue. Based on these results, we will focus on completion of the spatial correlation between HIFU-induced thermal dose and gene expression in the tissue mimicking phantom and combination of HIFU thermal ablation with heat-regulated IL-12 gene therapy to be tested in a 4T1 murine tumor model. If successful, this synergistic combination of HIFU thermal therapy and heat-inducible gene therapy can lead to a superior tumor suppression effect due to simultaneous reduction of primary tumor mass and significantly boosted therapeutic effects.

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P16-6: HYPERTHERMIA-INDUCED TUMOR METABOLIC CHANGE AND TUMOR REOXYGENATION

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Tumor hypoxia, which is caused by imbalance between O₂ supply and consumption, is related to poor prognosis and therapy resistance. Hyperthermia (HT) is a strong adjuvant cancer treatment because of its cell killing and radiosensitizing effects on both normoxic and hypoxic cells. HT, especially at mild temperatures between 39 and 43 degrees, induces tumor reoxygenation, which results in improved response to radiotherapy. It has been suggested that tumor reoxygenation is due to increased blood flow and decreased oxygen consumption. However, the underlying mechanism is not well understood yet.

Under hypoxia, cancer cells exhibit an adaptive mechanism via hypoxia-inducible factor-1 (HIF-1), a transcription factor that activates a variety of genes involved in tumor progression. In prior studies, our group has demonstrated that radiation-induced reoxygenation activates HIF-1 through reactive oxygen species (ROS), and inhibition of HIF-1 leads to the enhanced antitumor effects. Since HIF-1 is known to regulate

tumor metabolism by activating glycolytic enzymes and inhibiting mitochondrial functions, we hypothesized that HT-induced tumor reoxygenation is caused by the alteration of tumor metabolism, and HIF-1α plays a role in it.

To determine the effect of heat on HIF-1, we first heat-treated a mouse mammary carcinoma cell line (4T1) at a range of temperatures for 1 hour and measured HIF-1 levels using the luciferase reporter. This luciferase reporter contains the oxygen-dependent degradation (ODD) domain of HIF-1α, enabling us to directly detect HIF-1 stability. Interestingly, at mild hyperthermic temperatures, HIF-1 levels were significantly increased and were the highest at 42°C. Increased HIF-1 activity after heat treatment was also detected using HIF-1 ELISA and measuring VEGF secretion, a downstream target of HIF-1. The same experiments at different time points (0, 1, 2, 3, 6, 12, and 24 hours) showed that HIF-1 activity increased immediately and peaked 6 hours after heat shock. Our in vivo study growing the same reporter cell line (4T1) on the flank of nude mice confirmed our in vitro data. Plasma levels of PAI-1, another HIF-1 target, were significantly increased after HT. To determine the possible role of ROS in HIF-1 activation, we treated 4T1 cells with an SOD mimetic. Treatment with the SOD mimetic abolished the upregulation of HIF-1, indicating that ROS play a role in HIF-1 regulation after HT. Since mitochondrial ROS regulates HIF-1, the link between mitochondrial function and HIF-1 activity was next examined by measuring mitochondrial membrane potential with JC-1. JC-1 aggregates decreased 24 hours after HT, indicating the disruption of membrane potential.

In conclusion, our study showed that mild HT increased HIF-1 activity is related to mitochondrial ROS production. Thus, alteration of tumor metabolism by HIF-1 causes reduced oxygen consumption, and as a result, the tumor is reoxygenated. Future studies will include measurement of ROS production and glycolytic enzymes, as well as inhibition of HIF-1 using an HIF-1 knockdown cell line.

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P16-7: MAGNETICALLY LABELED TRANSGENIC ENDOTHELIAL PROGENITOR CELLS AND DENDRITIC CELLS AS PROBES FOR CELLULAR MRI AND GENE DELIVERY VEHICLES

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Cancer gene therapy is at a point where the need for an optimal gene delivery vector has become the rate-limiting step. Several factors, including lack of an efficient vector and delivery system itself, limit the effectiveness of systemically delivered genes. Very recently, stem/progenitor cells have been considered as delivery vehicles for transferring exogenous genes to the cancer cells. The purpose of this study was to determine whether endothelial progenitor (EPCs) and/or dendritic cells (DCs) can be used as gene delivery vehicles and as cellular probes for magnetic resonance imaging (MRI). In this study, we used superparamagnetic iron oxide (SPIO)-labeled EPCs and DCs to carry human sodium iodide symporter (hNIS) gene to the sites of implanted breast cancer. In vivo real-time tracking of these cells was performed by MRI and expression of hNIS was determined by Tc-99m pertechnetate (Tc-99m) scan.

Three million human breast cancer (MDA-MB-231) cells in 50 µL of serum free media were subcutaneously implanted in the right flank of nude mice. EPCs (CD34+/AC133+) were isolated from fresh human cord blood. DCs were made either from the cord blood CD34+ or CD14+ cells using established protocols. Both EPCs and DCs were genetically transformed to carry hNIS gene using adenoviral vectors. Genetically transformed EPCs and DCs were magnetically labeled with ferumoxides-protamine sulfate (FePro) complexes as previously published. Magnetically labeled genetically transformed cells were administered intravenously in tumor bearing mice. MRIs were acquired 3 or 7 days after cell injection and SPECT images were acquired within the 24 hours of performing the MRI. T2, T2*, and 3D GRE images were obtained using a 7 Tesla, 20 cm bore superconducting magnet interfaced to a BRUKER console. SPECT images were acquired with custom built micro-SPECT using Tc-99m. After SPECT, animals were euthanized, perfused, and whole tumors were collected for histochemistry.

Both cell types were efficiently magnetically labeled (>90%). MRI images clearly showed the presence of low signal intensity areas around the tumors in mice that received iron-labeled cells, indicating the accumulation of administered cells. The presence of iron-labeled cells was also confirmed by Prussian blue staining. In addition, SPECT images showed significantly higher radioactivity in tumors in animals that received transfected cells (p-value<0.01). The activity of Tc-99m in the tumors in 3 and 7 days post-injection transfected groups were significantly higher than that of control groups that received non-transfected cells (p-value<0.01). However, there were no significant differences between 3 and 7 day post-injection transfected groups. Immunohistochemistry proved the presence of hNIS positive cells in the tumors.

Both, MRI and SPECT images showed accumulation of administered EPCs and DCs in implanted breast cancer and expression of hNIS gene, respectively. Our study indicates that both EPCs and DCs can be used to deliver genes by systemic administration. Genetically transformed, magnetically labeled DCs or EPCs can be used both as delivery vehicles as well as cellular probes for detecting in vivo migration and homing of cells by MRI. This method can be used in the future development of gene therapy approaches where genetically modified cells can be tracked by real time in vivo MR scanning in different disease processes.

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P16-8: NOVEL BREAST CANCER THERAPEUTICS BASED ON BACTERIAL CUPREDOXIN

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Background and Objectives: Reports of regression of cancer in humans infected with microbial pathogens date back more than 100 years. However, live bacteria produce significant toxicity, limiting their use. The unprecedented observation that the small bacterial protein *Pseudomonas aeruginosa* azurin forms a complex with the well-known tumor suppressor protein p53 and triggers cell death provides a new avenue for cancer research. Despite being a novel concept to target cancer, there are no thermodynamic details known for the proposed azurin-p53 complex. This project aims to fill this gap as outlined in four specific aims.

Aims and Methodologies: We will reveal (1) which p53 domain interacts with azurin and probe affinity and stoichiometry, (2) the molecular mechanism by which azurin increases cellular levels of p53, (3) the region on azurin that interacts with p53 and (4) use the acquired information to propose smaller molecules that retain properties of azurin. For this, we use a battery of biophysical, spectroscopic and biochemical techniques in conjunction with purified proteins and strategic variants for in vitro experiments.

Results to Date: To this date, several discoveries have been made: most importantly, azurin is found to bind to the unstructured N-terminal domain of p53 and a small 13-residue peptide is able to reproduce part of the azurin interaction. Also, properties of two human copper-metabolism proteins have been identified; these proteins are important as they may cross-react with azurin-based drugs.

Conclusions and Impact: Our biophysical project provides key physical, chemical, and structural understanding of azurin's interaction with p53 in vitro. We propose that the results of our studies may be used to develop small peptide constructs that bind and stabilize p53 like full-length azurin. If these molecules turn out to work in vivo, it may be the gateway to an innovative class of new cancer therapeutics.

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P16-9: THE ROLE OF VITAMIN D IN AROMATASE INHIBITOR-INDUCED BONE LOSS

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Breast cancer (BCa) is the most common fatal cancer in women. Emerging evidence suggests that vitamin D is inversely related to BCa: low vitamin D levels are associated with increased BCa risk and vitamin D insufficiency is prevalent among women with BCa. Potent aromatase-inhibitors (AI) effectively suppress BCa growth by blocking local estrogen synthesis in the breast tissue, but they also cause severe side effects including hot flashes, muscle and joint pains, and bone loss leading to a substantially increased risk of fractures. These side effects are likely related to profound suppression of local estrogen production in bone and muscle tissues. Accumulating evidence indicates that higher than currently recommended doses of vitamin D are effective in the prevention and treatment of several cancers, including BCa. Preliminary data also suggest that vitamin D is potentially capable of reversing the AI-induced deleterious effect on bone and muscle. Human and animal experiments are currently under way to determine whether co-administration of vitamin D along with AIs is safe and effective in protecting patients with BCa from AI-induced increases in the rate of bone turnover that lead to bone loss and fractures, as well as preventing or ameliorating AI-induced muscle and joint pains. If vitamin D, this inexpensive and widely available therapeutic agent, is proven safe and effective in women with BCa, it will potentially open new avenues to investigate the role of vitamin D in prevention or treatment of BCa in the future.

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P16-10: TOPOISOMERASE II β AND DOXORUBICIN CARDIOTOXICITY

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Background and Objective: Doxorubicin (Adriamycin) is one of the most effective anticancer drugs used in the clinic for treatment of breast cancers. However, doxorubicin therapy can cause life-threatening cardiotoxicity. Despite the severity of this dose-limiting toxicity, the molecular mechanism underlying doxorubicin cardiotoxicity remains unclear. The free radical hypothesis is currently most favored due to the redox cycling ability of doxorubicin. However, this hypothesis is still controversial since many free radical scavengers fail to rescue doxorubicin cardiotoxicity. The only clinically used cardioprotective agent is dexrazoxane (also known as ICRF-187). It is well known that Top2 is the major cellular target of doxorubicin. The antitumor activity of doxorubicin is due to the formation of a Top2-doxorubicin-DNA ternary complex (the cleavage complex). However, there are two Top2 isozymes (Top2 α and Top2 β) in human cells. Both isozymes can be targeted by doxorubicin. Top2 α is only expressed in proliferating cells such as tumors. By contrast, Top2 β is expressed in terminally differentiated cells such as adult cardiomyocytes. We hypothesize that doxorubicin cardiotoxicity is primarily Top2 β -mediated. There are at least three lines of evidence supporting our hypothesis. First, the cardioprotective agent dexrazoxane is a known derivative of bis(2,6-dioxopiperazines) compounds that are also Top2 catalytic inhibitors. Top2 catalytic inhibitors are known to antagonize the formation of Top2 cleavage complexes. Second, Top2 β , but not Top2 α , is highly expressed in cardiomyocytes of the adult heart. Third, a C-terminal truncated form of Top2 β has been identified in mitochondria that are known to be abundant in cardiomyocytes.

The goal of this application is to rigorously test our hypothesis that the Top2 β isozyme is primarily responsible for doxorubicin cardiotoxicity.

Methodology: The role of Top2 β and proteasome in doxorubicin-induced DNA damage is investigated using primary TOP2 $\beta^{+/+}$ and TOP2 $\beta^{-/-}$ mouse embryonic fibroblasts (MEFs) and H9C2 cardiomyocytes, respectively. Doxorubicin-induced DNA damage signal γ -H2AX (by western blotting) and DNA double-strand breaks (DSBs) (by neutral comet assay) are monitored. In addition, a mouse model will be developed to determine the role of Top2 β in doxorubicin cardiotoxicity. Temporal-controlled heart-specific TOP2 β knockout mice will be generated by crossing the floxed TOP2 β mouse line with the transgenic mouse line expressing an α -MHC promoter-driven progesterone receptor/Cre fusion protein. Cre-mediated deletion of the TOP2 β gene will be achieved by the intraperitoneal (i.p.) injection of RU486. Cardiotoxicity induced by a single injection of doxorubicin will be monitored by transthoracic echocardiography, histological analysis, and TUNEL assay.

Results and Conclusion: We show that doxorubicin-induced DNA damage is much reduced in TOP2 $\beta^{-/-}$ MEFs, suggesting the involvement of Top2 β in doxorubicin-mediated cell killing. Proteasome inhibitors also reduce doxorubicin-induced DNA damage, suggesting the involvement of the proteasome pathway in doxorubicin cytotoxicity. These findings will be further tested in a mouse model using a conditional heart-specific TOP2 β knockout mouse line. Results from our study will have important implications in the clinic. If doxorubicin cardiotoxicity is indeed Top2 β -mediated, Top2 α -specific anticancer drugs should be developed to reduce side effects during chemotherapy.

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P16-11: A CHEMOTHERAPY-ASSOCIATED SENESENCE BYSTANDER EFFECT IN BREAST CANCER CELLS

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Virginia Commonwealth University

A bystander effect typically refers to the death, altered growth, or damage of cells that have not directly received chemotherapy or irradiation. Cancer cells derived from solid tumors readily undergo senescence in response to chemotherapeutic agents, prompting us to test for the existence of a senescence bystander effect. MCF-7 breast cancer cells were acutely exposed to adriamycin to trigger senescence. Naïve MCF-7 cells, when cultured in conditioned media from senescent breast cancer cells, growth arrested despite mitogenic stimulation and exhibited SA- β -galactosidase activity, an enlarged cell size, and stable upregulation of p21^{WAF1} protein, collectively indicating a senescent state. In contrast, HCT-116 colon cancer cells, which also undergo p53-mediated senescence in response to acute AdR, did not undergo growth inhibition or senescence when cultured with conditioned media from senescent HCT-116 cells. Reciprocal experiments indicated that naïve HCT-116 cells are susceptible to the growth inhibitory effects of a breast cancer-derived mediator, which is independent of drug in conditioned media. Our study reveals a novel action of adriamycin, which may contribute to its potent anti-breast cancer activity and lead to the discovery of additional therapeutic targets for the exploitation of a senescence bystander effect.

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P16-12: B CELL TRANSLOCATION GENE 1 CONTRIBUTES TO ANTISENSE Bcl-2-MEDIATED APOPTOSIS IN BREAST CANCER CELLS

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The antiapoptotic protein Bcl-2 is overexpressed in a majority of breast cancers and is associated with a diminished apoptotic response and resistance to various antitumor agents. Bcl-2 inhibition is currently being explored as a possible strategy for sensitizing breast cancer cells to standard chemotherapeutic agents. Antisense Bcl-2 oligonucleotides represent one method for blocking the antiapoptotic effects of Bcl-2. In this study, we show that antisense Bcl-2 efficiently blocks Bcl-2 expression, resulting in the apoptosis of breast cancer cells. Antisense Bcl-2-mediated cytotoxicity was associated with the induction of the B cell translocation gene 1 (BTG1). Importantly, knockdown of BTG1 reduced antisense Bcl-2-mediated cytotoxicity in breast cancer cells. Furthermore, BTG1 expression seems to be negatively regulated by Bcl-2 and exogenous expression of BTG1-induced apoptosis. These results suggest that BTG1 is a Bcl-2-regulated mediator of apoptosis in breast cancer cells and that its induction contributes to antisense Bcl-2-mediated cytotoxic effects.

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P16-13: INVOLVEMENT OF TOPOISOMERASE II β IN DEXRAZOXANE-MEDIATED PREVENTION OF DOXORUBICIN CYTOTOXICITY

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Background/Objective: Doxorubicin (Adriamycin), a DNA topoisomerase II (Top2)-targeting drug, is one of the most effective anticancer drugs used in the clinic. The antitumor activity of doxorubicin is due to the formation of a Top2-doxorubicin-DNA ternary complex (cleavage complex). However, doxorubicin-based chemotherapy could result in life-threatening cardiotoxicity. Despite the severity of this dose-limiting toxicity, the molecular mechanism underlying doxorubicin cardiotoxicity remains unclear. Currently, the free radical hypothesis is favored due to the redox cycling ability of doxorubicin to generate highly reactive oxygen free radicals. The cardioprotectant, dexrazoxane (also known as ICRF-187), is currently in clinical use to protect against doxorubicin toxicity. The mechanism for the protection has been primarily attributed to iron chelation by the EDTA-like hydrolysis product of dexrazoxane, which could decrease the level of hydroxyl-free radicals. However, the iron chelator ICL670A shows no cardioprotection against doxorubicin. On the other hand, dexrazoxane belongs to a class of molecules that are known to function as Top2 catalytic inhibitors. These compounds can antagonize the formation of Top2-DNA covalent (cleavage) complexes through their stabilization of the ATP-bound, closed-clamp conformation of Top2. In addition, recent studies have demonstrated that ICRF-193 (a bis(2,6-dioxipiperazines) derivative) can induce the preferential, proteasome-mediated degradation of Top2 β (termed Top2 β downregulation). It is currently unclear whether the cardioprotective effect of dexrazoxane involves Top2. There are two Top2 isozymes (Top2 α and Top2 β) in mammalian cells. Both isozymes can be targeted by doxorubicin. However, the two isozymes are regulated very differently. The high efficacy of doxorubicin chemotherapy is thought to be due to the highly elevated expression of Top2 α in cancer cells. By contrast, Top2 β is present in all cells including terminally differentiated postmitotic cells such as cardiomyocytes of the adult heart. It is possible that dexrazoxane-mediated cardioprotection could be related to the ability of bis(2,6-dioxipiperazines) compounds in inducing Top2 β downregulation in the heart, in which Top2 α is absent.

The goal of this proposal is to rigorously test our hypothesis that dexrazoxane protects cardiomyocytes against doxorubicin toxicity through its ability to attenuate doxorubicin-induced Top2 β cleavage complex formation and to induce Top2 β downregulation.

Methodology: The direct antagonizing effect of dexrazoxane in doxorubicin-induced DNA damage in H9C2 cardiomyocytes is investigated. The induction of the DNA damage signal γ -H2AX is monitored by the western blotting analysis. The indirect antagonizing effect of dexrazoxane, through Top2 β downregulation, in doxorubicin-induced DNA double-strand breaks (DSBs) is also investigated using the neutral comet assay.

Results and Conclusion: In this study, we show that dexrazoxane can specifically abolish the DNA damage signal, γ -H2AX, induced by doxorubicin, but not camptothecin or hydrogen peroxide, in H9C2 cardiomyocytes. Furthermore, in addition to antagonizing Top2 cleavage complex formation, dexrazoxane can also induce rapid proteasomal degradation of Top2 β , which parallels the reduction of doxorubicin-induced DNA damage. Together, our results suggest that dexrazoxane antagonizes doxorubicin-induced DNA damage through its interference with Top2 β .

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P16-14: THE ROLE OF AUTOPHAGY IN DOXORUBICIN-MEDIATED CYTOTOXICITY

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Autophagy, a membrane trafficking process leading to lysosomal degradation of cytosolic components, is implicated in tumor suppression. Heterozygous deletion of an essential autophagy gene *beclin1* increases tumor rates in mice. Compromised autophagy in humans as a result of monoallelic deletion of *Beclin1* is associated with ~50% sporadic breast cancers. Autophagy activation is a pro-survival mechanism in response to various stress conditions, including metabolic stress such as starvation and genotoxic stress such as DNA damage. However, excessive autophagy may also lead to autophagic cell death or type II programmed cell death. The exact role of autophagy in cancer chemotherapy is not clear. We proposed to investigate the impact of autophagy activation on the efficacy of doxorubicin, one most commonly used breast cancer chemotherapeutic agent. In the present study, mouse embryonic fibroblasts (MEFs) from the wild-type mice (*atg5*^{+/+} mice) or the mice with a targeted deletion of the essential autophagy gene *atg5* (*atg5*^{-/-} mice) were immortalized with expression of a dominant negative p53 gene and the E1A gene. The isogenic wild-type and autophagy-deficient cells were treated with doxorubicin, and the cytotoxicity was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays and the clonogenic assays. The *atg5*^{-/-} cells exhibit increased sensitivity toward doxorubicin treatment comparing to their wild-type counterparts. These mutant cells are more prone to apoptosis upon doxorubicin treatment, which likely contributes to the increased cytotoxicity. In addition, the paired isogenic wild-type and autophagy-deficient cells were treated with doxorubicin with or without the co-treatment of hydrochloroquine, an FDA-approved drug that has inhibitory effect on autophagy, and the cytotoxicity was measured by clonogenic assays. Hydrochloroquine enhances the cytotoxicity of doxorubicin, and its effect is partially dependent on the autophagy status of the cells. These results suggest that inhibition of autophagy can enhance the efficacy of doxorubicin. Currently we are generating the relevant mouse mammary tumor model by breeding the *beclin1*^{+/+} mice with the MMTV-ras transgenic mice and the role of autophagy in breast cancer chemotherapy with doxorubicin will be further determined in vivo using this model. Our research would eventually provide preclinical data that will help determine (1) whether *Beclin1* deletion in human breast cancers can serve as a prognostic marker and (2) whether a combinatory chemotherapeutic regimen with autophagy inhibitors such as hydrochloroquine can enhance the efficacy of doxorubicin.

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P16-15: SPECIFIC, REVERSIBLE CYTOSTATIC PROTECTION OF NORMAL CELLS AGAINST NEGATIVE EFFECTS OF BREAST CANCER THERAPY

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The treatment of cancer with chemotherapy has severe side effects that damage healthy proliferating cells such as hematopoietic precursors, hair follicle cells, and the epithelial lining of the intestine. These side effects often limit the doses of chemotherapy administered, allowing tumor cells to gain a growth advantage by escaping treatment and selecting for drug-resistant clones. The nonspecific nature of these agents targets all proliferating cells and tissues. Therefore, all cells that proliferate, whether normal or tumor, are affected by the treatment. However, if the normal dividing cells in the body were to stop proliferating reversibly, the toxic effects of chemotherapy would potentially be diminished. Two cytostatic agents (UCN-01 and staurosporine) have been previously shown in vitro in our laboratory to selectively and reversibly arrest normal dividing cells in G1. Tumor cells, which lack a functional restriction point in the cell cycle, are not affected by drug concentrations that arrest normal cells. Subsequent treatment of these arrested cells with chemotherapeutic agents was shown to be much less efficacious than that of untreated (proliferating) cells. Tumor cells, which do not undergo this reversible arrest, were also not protected from the cytotoxicity of the chemotherapeutics. The goal of this work is to demonstrate this effect in vivo using immunodeficient (nude) mice. Our hypothesis is that arrested normal cells will evade the toxic side effects of chemotherapeutic agents resulting in improved treatment modalities for cancer. We have developed a model system using bromodeoxyuridine (BrdU) labeling of the small bowel epithelial cells to follow changes in cell cycle kinetics due to cytostatic treatment. In addition, fluorescent labeling of intestinal sections has been used to visualize changes in the migration of dividing cells from the crypt to the villus. To date, we have been able to demonstrate a reversible arrest in the small bowel epithelium of the mouse following UCN-01 treatment. These cells undergo a two-fold increase in mean doubling time within 48 hours of treatment; by 2 weeks, the cells of the gut emerge from this arrested state and display kinetics similar to untreated mice. Future experiments will compare the toxic effects of several chemotherapeutic agents in these "protected" mice to those in mice not pretreated with UCN-01. We are also in the process of determining the mechanism for the temporary cell cycle arrest. It is hoped that the reversible arrest of

normal dividing cells will protect the host against the limiting toxic effects of chemotherapeutics while simultaneously allowing for the efficient treatment of tumors.

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P16-16: DIFFERENTIAL IMPACT OF ANTI-DIABETIC TREATMENTS ON BREAST CANCER CELL GROWTH IN CELL CULTURE CONDITIONS MIMICKING DIFFERENT STAGES IN THE NATURAL HISTORY OF DIABETES MELLITUS TYPE 2

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Background: Diabetes mellitus type 2 (DM2) is well known to be associated with increased risk as well as adverse outcome of breast cancer. Despite early investigations into mechanisms linking DM2 and breast cancer, only limited studies focused on translating this knowledge into potential clinical interventions that could modify the risk or outcome of diabetic breast cancer patients.

Methods: We investigated the impact of two interacting factors that change at various stages of the DM2 disease process (hyperglycemia and hyperinsulinemia) on the proliferation of breast cancer cells and examined whether some antidiabetic drugs, namely metformin, rosiglitazone, and exenatide, have direct inhibitory effects on MCF7 cells in vitro.

Results: Our data demonstrated that insulin promoted the proliferation of MCF7 breast cancer cells, but the growth-promoting effect of glucose was dependent on insulin and was observed only at high insulin concentrations. Both insulin and glucose contributed to the chemoresistance of breast cancer cells to doxorubicin. Treatment with metformin or rosiglitazone suppressed MCF7 cell growth via inactivation of the Akt pathway, leading to apoptosis. Metformin, a biguanide antidiabetic drug, activated AMPK, and rosiglitazone, a PPAR- γ agonist, increased the protein level of PTEN. The inhibitory effects of these two drugs were observed regardless of the various combinations of glucose and insulin concentrations mimicking different stages in the natural history of DM2. Both metformin and rosiglitazone resulted in further inhibition of MCF7 cell growth and increase in apoptosis when combined with doxorubicin in all the combinations of glucose and insulin concentrations tested. Exenatide, an incretin analog, did not have any inhibitory effects on MCF7 cells nor did it improve chemosensitivity when combined with doxorubicin.

Conclusion: Our results showed that either culture conditions mimicking hyperinsulinemia and hyperglycemia stimulated breast cancer cell proliferation and conferred chemoresistance to doxorubicin. Two antidiabetic drugs commonly used for DM2, metformin and rosiglitazone, had direct effects on MCF7 cells and added to the inhibitory effect of doxorubicin. These results have significant therapeutic implications in terms of the choice of pharmacotherapy for DM2 in the context of diabetic breast cancer patients that warrant further investigation in animal studies.

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P16-17: ZOLEDRONIC ACID AND PTH INCREASE BONE MASS AND MECHANICAL STRENGTH FOLLOWING RADIATION THERAPY FOR OSTEOLYTIC BONE METASTASES

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Radiation therapy (RTX) is highly effective in controlling bone pain in patients with osteolytic bone metastases. Unfortunately, these patients are still at high risk of developing pathological fractures at the site of the tumor. Although bisphosphonates (BPs) have been shown to reduce skeletal morbidity, their effects on the mechanical properties of irradiated bone have not been established. The specific aim of this study was to quantify the effects of a clinically proven BP (zoledronic acid, ZA; Novartis Pharma AG, Basel) on bone mass, microarchitecture, and mechanical properties following radiation therapy for an osteolytic bone metastasis. Additionally, we sought to determine if the beneficial effects of BPs could be further enhanced by concurrent use of an anabolic agent, PTH 1-34, following irradiation. The right distal femur of 21 female nude mice were injected with 1×10^5 F10 human breast cancer cells. Mice were divided into one non-irradiated control group (0Gy, n=4) and three treatment groups: 20Gy only (20Gy, n=5), 20Gy plus ZA (ZA, n=6), 20Gy plus PTH and ZA (PTH/ZA, n=6). RTX was administered to the right femur 3 weeks post-tumor inoculation. ZA (100 μ g/kg SC) was administered once weekly for 6 weeks starting 3 days before irradiation and PTH (80 μ g/kg SC) was administered 5 days a week for 4 weeks starting the day after irradiation. Radiographs and DEXA scans were obtained for each animal at 3, 6, and 9 weeks post-tumor inoculation. All animals were eutha-

nized at 9 weeks or earlier if severe lameness or pathology occurred. The hind limbs from all animals were explanted, scanned by micro-CT (5 μ m resolution) and mechanically tested in torsion through the intact knee joint. ANOVA and Fisher's PLSD statistical tests were performed to analyze comparisons between groups. Dramatic increases in bone volume (55%, p=0.0012) and bone mineral density (28%, p=0.0037) were seen in mice treated with ZA compared to those treated with 20Gy only. Similar improvements (64% increase in BV, p=0.0012; 33% increase in BMD, p=0.0001) were seen in mice treated with the dual drug protocol. Micro-CT analysis revealed that although ZA alone did not lead to a significant increase in fractional femoral trabecular bone volume (BV/TV) as compared with 20Gy only (p=0.7), dual treatment increased BV/TV by over 300% as compared with 20 Gy only (p=0.007). Treatment with PTH/ZA or ZA significantly increased the mechanical strength of irradiated femora by 117% (p=0.04) and 115% (p=0.013), respectively. These results clearly demonstrate that the beneficial effect of BPs on bone mass and microarchitecture led to clinically relevant increases in the mechanical properties of bones following radiation therapy. The current clinical practice of BP therapy for patients with osteolytic bone metastases therefore appears to be entirely appropriate but additional gains in bone mass and microarchitecture should be attainable if BPs are combined with an anabolic agent. Given the concerns over the use of PTH 1-34 in cancer patients, our future work will focus on using this mouse model to screen the safety and efficacy of additional candidate anabolic agents for the treatment of osteolytic bone metastases.

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P16-18: BUILDING A BETTER MOUSE FOR HUMAN BREAST CANCER DRUG TESTING

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Despite enormous expenditures and efforts by academic, government, and pharmaceutical institutions over many years, most breast cancer drugs that show promise in mice fail to cure breast cancer in patients. Such an inefficiency is enormously costly and hampers identification of new and effective breast cancer drugs. This discrepancy suggests that mice are not a reliable model for correct prediction of drug responsiveness of human breast cancer.

Nonetheless, before candidate drugs against breast cancer are allowed into clinical trials, FDA requires promising effects on human breast cancer grown in immunodeficient mice. The mouse host is considered to provide an appropriate hormonal environment for mimicking breast cancer growth as it occurs in the human. Three cardinal hormones, estrogen, progesterone, and prolactin, regulate growth of breast cancer. Importantly, most human breast cancers retain prolactin receptors, prolactin is present in serum of both pre- and postmenopausal women, and prolactin stimulates growth, survival, and motility of human breast cancer cells.

We made the serendipitous discovery that human prolactin receptors are not only insensitive to mouse prolactin, but mouse prolactin acts as an antagonist toward human prolactin receptors. Consistent with this, prolactin-induced signaling pathways are effectively shut off in human breast cancer grown as xenotransplants in mice even when human breast cancer cells produce prolactin in an autocrine manner. This realization led us to postulate the novel concept that the mouse hormonal environment does not mimic the hormonal environment in patients. Consequently, the biological behavior and drug responsiveness of a breast tumor will be different once the tumor is transferred from a patient into mice.

We have successfully created genetically engineered mice that express human prolactin instead of mouse prolactin and crossed the mice into an immunodeficient background. Mice expressing physiological levels of human prolactin were achieved fulfilling the objectives of the concept award. Current studies are testing the new mice as recipients for human breast cancer cell lines and tissue explants. The new mouse model is projected to have several advantages: (1) provide improved modeling of human breast cancer under more relevant hormonal conditions with superior growth environment for both established metastasis-derived lines and new lines derived directly from primary tumors, (2) provide more reliable preclinical drug testing and better prediction of drug responsiveness in the clinic, and (3) provide a unique model that will for the first time allow testing of therapeutic targeting of prolactin receptor pathways. Establishment of new and more relevant transplantable human breast cancer lines and more reliable preclinical drug testing would facilitate identification of new and effective breast cancer drugs. In addition, individual testing of patient tumors in the new host for responsiveness to existing drugs is expected to improve individualized tailoring of medicine for breast cancer patients.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0512.

P16-19: GENETICALLY INDUCED TUMORIGENESIS IN SWINE**Brooke Ancrile,¹ Kristy Kuzmuk,² Laurie Rund,² James Zachary,² Lawrence Schook,² Christopher Counter,¹ and Stacey Adam¹**¹Duke University Medical Center and ²University of Illinois, Champaign/Urbana

Animal models are invaluable in preclinical cancer studies; however, mice, the most commonly used models, present limitations for preclinical studies in imaging, hyperthermia, photodynamic, and radiation therapies. Therefore, we sought to exploit the pig as an experimental and preclinical model for human cancer. Pigs offer the advantage of having a similar size, diet, metabolism, and anatomy to humans. To this end, we show that multiple primary porcine cell strains can be genetically converted to a tumorigenic state, as assessed in immunocompromised mice, by expression of genes known to promote tumorigenic growth of human cells. Moreover, when such cells are returned to the host animal, tumors develop. However, this tumorigenic growth

depends on suppression of the immune system, likely owing to epigenetic and antigenic changes in the cells during culture manipulation. Further studies determined that in vivo tumor induction methods employed can drive selected cell types—mainly lymphocytes—to malignant fates and overcome the need for immunosuppression. Future experiments will focus on increasing the reproducibility of the in vivo tumor induction, as well as exploring the possibility of transforming other cell types in vivo to produce other cancer types. In summary, we have developed two rapid, reproducible, and genetically malleable methods to induce tumors in swine similar to those treated clinically in humans. This will provide a robust preclinical model for the study of imaging, hyperthermia, photodynamic, and radiation therapies in the treatment of human cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0437.

IMMUNE-BASED THERAPIES I

Poster Session P17

P17-1: TELOMERASE-SPECIFIC T-CELL IMMUNITY IN BREAST CANCER: EFFECT OF VACCINATION ON TUMOR IMMUNOSURVEILLANCE

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Background: The human telomerase reverse transcriptase (hTERT) is nearly universally overexpressed in human cancer, contributes critically to oncogenesis, and is recognized by cytotoxic T cells that lyse tumors. CD8+ T cells specific for hTERT naturally occur in certain populations of cancer patients in remission, but it remains poorly understood whether such T cells could contribute to tumor immunosurveillance. To address this issue, we performed several studies investigating approaches to hTERT vaccination in metastatic breast cancer patients.

Methods: We report the immunologic and biologic effects of vaccinating metastatic breast cancer patients with hTERT peptide. Nineteen HLA-A2+ women with metastatic breast cancer and who had no measurable T-cell response to hTERT at baseline were vaccinated with hTERT 1540 peptide in adjuvant with GM-CSF for up to 8 vaccinations. Additional studies have been initiated examining immunomodulation prior to hTERT vaccination either with a single dose of cyclophosphamide (n=5) or a single dose of daclizumab (n=3 to date, study is actively accruing patients).

Results: Tumor-infiltrating lymphocytes (TIL) were evident after, but not before vaccination, with 4% to 13% of postvaccine CD8+ TIL specific for the immunizing hTERT peptide. Induction of TIL manifested clinically with tumor site pain and pruritus and pathologically with alterations in the tumor microenvironment, featuring histiocytic accumulation and widespread tumor necrosis. hTERT-specific CD8+ T cells were also evident after vaccination in the peripheral blood of patients and exhibited effector functions in vitro including proliferation, IFN-gamma production, and tumor lysis. An exploratory landmark analysis revealed that median overall survival was significantly longer in those patients who achieved an immune response to hTERT peptide (32 months) compared with patients who did not (17.5 months). Immune response to a control cytomegalovirus peptide in the vaccine did not correlate with survival.

Conclusions: These results suggest that hTERT-specific T cells could contribute to the immunosurveillance of breast cancer and suggest novel opportunities for both therapeutic and prophylactic vaccine strategies for cancer. Ongoing studies are investigating whether immunomodulation prior to vaccination will enhance immune response.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0619 and National Institutes of Health (R01 CA-111377).

P17-2: ENHANCED ANTITUMOR IMMUNIZATION BY LIPOSOMAL DELIVERY OF VACCINE

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A key objective in vaccination strategies is to overcome the body's immune tolerance of tumor cells. One approach to achieving this objective is to present a tumor-specific molecule in the context of an adjuvant—that is, a cytokine or other activator of the immune system. Liposomal vaccines have been previously described, but these have generally been of the order of 100 nm in diameter. We have observed that large, 650 nm-diameter PEG-liposomes, injected IV, localize to the spleen rapidly (i.e., they are splenotropic) and at very high concentration. The spleen contains the most potent cells for the processing and presentation of antigens for activation of naïve T-lymphocytes. Splenotropic liposomal vaccines have been investigated as a means of enhancing vaccination against breast cancer. We developed 600 to 800 nm liposomal constructs containing the RNEU peptide and GM-CSF and examined their localization within the spleen. We examined dendritic cell uptake following IV administration of different liposomal constructs stained with FITC for flow cytometry detection. The different liposomes were administered at day zero and 4, and the mouse was sacrificed 4 days after the last injection (day 8); the spleen was extracted rapidly and the cells dissociated. Dendritic cells were isolated for flow cytometry by a magnetic separation technique. Dendritic cell uptake of non-PEG liposomes with or without the immunostimulatory peptide RNEU was less than 2%. Dendritic cell uptake of PEG liposomes was approximately 4%. No difference was observed for PEG constructs with RNEU in the hydrophilic interior of the liposomes versus placing the peptide on the surface of the PEGylated liposome. T-cell activation was not observed, however, and the work is continuing with alternative liposomal vaccine formulations.

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P17-3: DESIGNING CARBOHYDRATE MIMETIC PEPTIDES AS BROAD SPECTRUM IMMUNOGENS

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Purpose: Maximizing sustained antibody immunity against Tumor-Associated Carbohydrate Antigens (TACAs) is an important goal in developing effective cancer vaccines to combat recurrent disease. We previously demonstrated that carbohydrate mimetic peptides (CMP) 106 (GGIYWRDYIYWRDYIYWRDY) and 107 (GGIY- YRYDIYRYDYIYRYDY) mimic complex carbohydrates like LeY and simple mono- and disaccharide, components of a variety of TACA. This multiple antigen mimicry (MAM) provides power to these CMP in that they preclude the development of multi-valent vaccines encompassing multiple TACA. To further develop such CMPs that target a broad spectrum of TACA, we are evaluating a candidate peptide, P10s (WRYTAPVHLGDG), as a LeY/ganglioside mimotope. P10s possesses in its sequence the WRY motif, also present in the peptide 106. We observed that P10s peptide binds to an anti-LeY mAb, BR55-2 and to anti-GD2/GD3 antibodies. Thus, P10s antigenically mimics the TACAs that are expressed on breast cancer cells.

Experimental Design: Utilizing crystal structures, molecular models of anti-TACA reactive antibodies and surface plasmon resonance we are testing the hypothesis that improving the hydrogen bond pattern of CMP to be coincident with that for the carbohydrate ligand, will enhance the ability of these peptides to elicit TACA reactive antibodies.

Results: The monoclonal antibody ME36.1 shows broad specificity in reacting with gangliosides. Primary hydrogen bonding partners of ME36.1 binding with GD2 suggests that ME36.1 could react with a spectrum of gangliosides expressed on tumor cells. We developed a peptide with the sequence WRYTAPVHLGD (referred as P10s) that shares 5 hydrogen bonds with GD2 in binding to ME36.1. Docking calculations indicate that the topographical binding mode of P10s overlaps that of GD2 in the ME36.1 combining site. Importantly we observe that P10s also displays increased reactivity with LeY reactive monoclonal antibody. Modified peptide P10s shows enhanced ability to induce anti-GD2/LeY humoral immune response. Immunization with P10s induced serum IgM antibodies superior in GD2/LeY binding than serum antibodies induced by analog CMPs of P10s. We observed a significant increase in survival time of mice immunized with P10s.

Conclusions: These data indicate that we can develop novel CMPs based upon rational design concepts that induce anti-tumor responses.

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P17-4: ABSTRACT WITHDRAWN

P17-5: ANTI-TUMOR IMMUNIZATION BY LIPOSOMAL DELIVERY OF VACCINE TO THE SPLEEN

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A key objective in vaccination strategies is to overcome the body's immune tolerance of tumor cells. One approach to achieving this objective is to present a tumor-specific molecule in the context of an adjuvant, that is, a cytokine or other activator of the immune system. Liposomal vaccines have been previously described, but these have generally been of the order of 100 nm in diameter. This proposal was based on the following observations: (1) that large, 650 nm-diameter PEG-liposomes, injected iv, localize to the spleen rapidly and at very high concentration. Splenic localization is highly dependent upon the size distribution of the liposomes with smaller liposomes (100 and 400 nm diameter) not showing the level of localization and retention seen with larger liposomes and (2) that the spleen contains the most potent cells for the processing and presentation of antigens for activation of naïve T-lymphocytes.

The specific aims of this proposal were to develop large, 600 to 800 nm diameter, PEGylated liposome vaccine constructs. Six types of liposome constructs with the (1) antigenic rat HER2/neu epitope, RNEU420-429 and adjuvant (e.g., GM-CSF) in the hydrophilic interior, (2) lysates of 3T3 cells (engineered to overexpress *neu* and to secrete GM-CSF) in the hydrophilic core and (3) lysates of NT2 cells and GM-CSF in the hydrophilic core were developed with CpG oligonucleotides either on the surface or in the core. Splenic localization and biodistribution/fate of the various vaccine liposomes, in vivo, T cell activation in neu transgenic mice and the efficacy of various liposome constructs with whole-cell vaccination in vivo will be evaluated.

Large liposomes were produced by the bath sonication method. RNEU₄₂₀₋₄₂₉ with GM-CSF were passively loaded into the hydrophilic interior of the liposome with either CD4 helper epitopes (CpG oligonucleotides) within the core or on to pegylated liposomes that were surface localized by standard means. Biodistribution and imaging studies will be performed using various liposome vaccine constructs passively loaded with In-111 and imaged by microSPECT/CT. Quantitative biodistribution will be

obtained by sacrificing the animals and extracting tissues for gamma counting. Microdistribution and fate of all six liposome vaccine constructs will be obtained by optical, fluorescent microscopy/imaging *ex vivo* of fluorescein-tagged liposome vaccine constructs. T cell activation studies were performed by intracellular cytokine assay, and efficacy studies were performed using the *neu-N* transgenic mouse model.

Immunotherapy in the form of antitumor vaccination has yielded occasional but not consistently promising results. This treatment modality is particularly attractive since the toxicity is negligible compared to conventional treatments such as chemotherapy. Breast cancer, due to its long latency period and metastatic spread, is an ideal cancer for a minimally toxic vaccine treatment approach. This proposal favors development of a novel vaccination approach that delivers the vaccine to those cells in the body that are most effective at activating the immune system and mounting an antitumor reaction.

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P17-6: DEPLETION OF CD4+CD25+ T REGULATORY CELLS AND LOCALIZED IRRADIATION IMPROVED EFFICACY IN COMBINATION TREATMENTS OF ALPHA-PARTICLE RADIO-IMMUNOTHERAPY AND WHOLE CELL CANCER VACCINE

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Cancer vaccine is a promising systemic therapy that activates host adaptive immunity and recruits cytotoxic T cells to eradicate tumor cells. However, tumor induces several immune suppressive mechanisms to inhibit potent T cell responses activated by cancer vaccines. It is, therefore, desirable to enhance the anti-tumor immunity by overcoming tumor immunosuppression within the tumor microenvironment. Alpha particle radioimmunotherapy is a potential approach to overcoming tumor immunosuppression. Pretreatment with alpha particle-labeled monoclonal antibody (mAb) reduces the number of tumor cells actively involved in immunosuppression and causes cancer cell lysis that releases multiple cytokines that may change the balance of these molecules within the tumor microenvironment. These changes may activate suppressed adaptive immunity and thereby enhance subsequent cancer vaccine treatment. To test this hypothesis, systemic treatment (i.v. injection) of ²¹³Bi labeled anti-rat HER-2/*neu* monoclonal antibody (7.16.4) in combination with GM-CSF secreting whole cell vaccine directed at HER-2/*neu* was tested in both a s.c. implanted tumor model and a metastatic tumor model induced by left cardiac ventricle injection of tumor cells in immunotolerant *neuN* mice. Although both groups have seen tumor growth inhibition and improved survival by ²¹³Bi labeled 7.16.4, no synergistic efficacy was observed from the subsequent vaccine treatment. Since systemic treatment by ²¹³Bi-7.16.4 showed evident immune suppression as measured by the decrease in T lymphocyte population, the vaccine treatment was scheduled to start when the T lymphocyte population had rebounded to above 80% of the pretreatment level. However, the weak T cell response following cancer vaccine inoculation suggested that such radiation induced immune suppression might have a negative impact on the ability of immune system to launch an effective immune response. Following these observations, we tested the efficacies of combining the two treatment modalities using localized tumor irradiation with these groups: (a) untreated controls; (b) cyclophosphamide (100 mg/kg) + whole cell vaccine; (c) ²¹³Bi-7.16.4Fab' (120 μ Ci) s.c. injection; (d) ²¹³Bi-7.16.4Fab' (120 μ Ci) s.c. injection + whole cell vaccine; (e) ²¹³Bi-7.16.4Fab' (120 μ Ci) s.c. injection + cyclophosphamide (100 mg/kg) + whole cell vaccine. When s.c. ²¹³Bi-7.16.4 treatment was combined with vaccine alone, no significant improvement (c and d) was found in tumor inhibition. When cyclophosphamide was included to deplete CD4+CD25+ T regulatory cells before vaccination, statistical significant improvement in tumor growth inhibition was found between combination treatment (e) and ²¹³Bi-7.16.4Fab' treatment alone (c) or the vaccine treatment without using cyclophosphamide (d), with a tumor size of 15.0 \pm 12.9, 43.0 \pm 8.2, 36.5 \pm 7.5 mm² at day 36 after tumor inoculation, respectively. These results suggested that intact immune system and depletion of CD4+CD25+ T regulatory cells are necessary to improve tumor growth inhibition by combination treatment of alpha particle radioimmunotherapy and cancer vaccine.

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P17-7: EXPRESSION, FUNCTION, AND VACCINE POTENTIAL OF PDEF IN BREAST CANCER

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Previous work from our lab has shown frequent overexpression of PDEF (prostate derived Ets transcription factor) in human breast tumors (Sood AK. et al., *Human Pathology*, 38:1628-1638, 2007). We now find that transfection of PDEF into MCF-12A breast epithelial cell enhances its tumorigenic growth in immunodeficient mice (our unpublished data). Further, downregulation of PDEF by an anti-sense oligonucleotide in MCF-7 breast tumor cell line reduced its viability *in vitro*. These observations further support a role for PDEF in breast tumor progression. To identify the downstream target genes regulated by PDEF, HG-U133A gene chip from Affymetrix was screened with labeled RNA probes from MCF-7 and from PDEF-downregulated MCF-7 cells. Genes with 2-fold or higher change in expression were identified. Our data show that 62 genes were upregulated at 2-fold or higher levels in PDEF-positive MCF-7 cells in comparison to PDEF-downregulated MCF-7 cells. In the same comparison, another 53 genes were found downregulated at 2-fold or higher levels in the PDEF-positive MCF-7 cells. The genes with 3-fold or higher altered expression levels were selected for further analysis by quantitative PCR. From these analyses, some of the PDEF-upregulated genes included alpha N-Catenin, CEACAM-6, CXCR4, S100-A7, and B7-H4, and those that were downregulated by PDEF included carbonic anhydrase XII and TNFSF10. The functional characteristics of these genes are consistent with their role in breast tumor growth and progression. Together, our results support the thesis that PDEF overexpression is causally related to breast tumor progression, and they support PDEF as a highly desirable target for developing novel therapeutics against breast cancer.

The previous work from our and other labs also showed that PDEF expression is highly restricted in normal human tissues, which is primarily limited to normal prostate with weak expression in trachea. These expression characteristics suggested that PDEF could be immunogenic in females. To that end, we have evaluated the immunogenicity of the mouse homologue of PDEF (Pse, prostate specific Ets) in mice. Our data show that immunization with Pse induces strong T cell responses in female mice but weak or little response in male mice. These results are novel and represent the first report of the gender-associated immunogenicity of an autosomal physiologic self-protein in female mice. This work has implications for testing PDEF as a novel vaccine target against breast cancer.

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P17-8: CANCER VACCINES TARGETING DEATH RECEPTOR DR5 FOR TNF-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL)

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Engagement of TRAIL with cognate death receptors (DR) induces apoptotic cell death in some cancers, but TRAIL-resistant cancers are often encountered. Phase I clinical trials with mAb to human DR4/5 resulted in stable disease in some cases. We hypothesize that induction of anti-DR Ab by active vaccination will provide protective antitumor immunity either alone or in combination with TRAIL therapy. To test this hypothesis, BALB/c mice were depleted of regulatory T cells (Treg) and immunized by intramuscular electro-vaccination with plasmid DNA encoding the extracellular and transmembrane domains of mouse DR5 (mDR5) fused at the C-terminus with domain 1 of tetanus toxin fragment C (pmDR5ectm-Td.1) or with xenogeneic SKOV-3 cells transfected to express mDR5ectm-Td.1. Anti-mDR5 Ab was detected by flow cytometry via specific binding to D2F2 mouse mammary tumor cells transiently transfected with pmDR5ectm/GFP encoding both mDR5ectm and eGFP; therefore, transfected cells expressing the recombinant mDR5 also expressed eGFP. Binding of immune sera to D2F2-mDR5/GFP cells with green fluorescence was detected to indicate the presence of anti-mDR5 Ab. Anti-mDR5 mAb MD5-1 was the positive control binding 100% of the cells that expressed the transgene. Electro-vaccination with heterologous human or rat DR5 DNA did not induce anti-mDR5 Ab.

Induction of tumor cell death was tested *in vitro* by incubating mammary tumor cells with agonist mAb MD5-1, mouse sTRAIL, or a combination of both. Neither MD5-1 nor sTRAIL induced cell death, but engagement of tumor cells with DR5 agonist MD5-1 prior to treatment with sTRAIL resulted in a robust apoptotic effect. Apoptosis was further enhanced by the addition of histone deacetylase (HDAC) inhibitor MS-275. Since TRAIL has been implicated in mediating immune surveillance and thymocyte apoptosis, DR5 expression in T cells activated by anti-CD3 and IL-2 was measured, and significant expression was detected by flow cytometry. Treatment of activated T cells with MD5-1 prior to the addition of TRAIL resulted in apoptosis; however, higher concentration of TRAIL was required to induce T cell death than that for tumor cell death.

To test the activity of DR5 as a vaccine, BALB/c mice electro-vaccinated with pmDR5ectm-Td1 and pGM-CSF four to five times following Treg depletion were challenged s.c. with mammary tumors D2F2 or TUBO. Both tumor lines express endogenous mDR5 at modest levels. Approximately 30% of immunized mice were protected from the challenge, suggesting that anti-mDR5 Ab induced by DNA vaccination may inhibit tumor growth. The efficacy and mechanisms of antitumor activity induced by DR5 vaccination with or without TRAIL therapy are being defined, and the potential benefit of combining chemotherapeutics in this regimen will be evaluated.

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P17-9: EVALUATION OF HUMAN IMMUNE RESPONSE TO MAITAKE BETA-GLUCAN IN A PHASE I STUDY IN BREAST CANCER PATIENTS

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Background: Maitake beta-glucan (MBG) is an extract from fruit body of *Grifola frondosa* mushroom that has been used to treat breast cancer and other malignancies in Asia. MBG contains glucan polysaccharide compounds that have a beta-1,6 glucopyranoside main chain with branches of beta-1,3 linked glucose. Although branched 1,3-beta-D-glucans have been used as biological modifiers in cancer in Asia, the mechanisms of action and dose relationships have not been previously defined in human. We used a systematic approach to model human immune response to MBG in vitro and then studied the effect in vivo on response ex vivo in a Phase I/II trial in breast cancer patients postchemotherapy.

Methods: Flow cytometric techniques were used to evaluate the effects of MBG on peripheral blood mononuclear cells (PBMC) including assessment of regulatory subsets, intracellular cytokine (IC) responses, and effector function. Secreted cytokines were assessed by ELISA. Cell proliferation and cytotoxicity assays were also carried out. A Phase I/II dose escalation study was conducted in five dose groups (0.2; 1; 3; 6; 10 mg/kg/day), 6 patients in each group. Stage II breast cancer post resection and chemotherapy patients were recruited. Patients self-administered oral doses of MBG twice daily for 3 weeks. Double baseline studies ex vivo were obtained pretreatment and then over the subsequent treatment weeks. Evaluation of immune response included immune cell subsets, production of monocyte and granulocyte respiratory oxidative species (ROS) in the burst test, IC response, and circulating cytokine.

Results: MBG had no direct cytotoxic or cytostatic effects on human PBMC, MCF-7 breast cancer, PC-3 prostate cancer cell lines, monocyte lines U937 and THP-1 by cell proliferation and cytotoxicity assays. Importantly MBG did not interfere with the cytotoxic effects of chemotherapy drugs Paclitaxel (Taxol®) and Docetaxel (Taxotere®) on MCF-7 cells. MBG activated IL-10 response of PBMC in vitro in IC assay. Also, MBG moderately enhanced Herceptin mediated (antibody dependent cell-mediated cytotoxicity) ADCC of PBMC towards MCF-7 cells. In the Phase I/II trial, baseline studies showed that breast cancer patients had altered lymphocyte subsets compared to normal controls with reduced CD3⁺ T cells, CD8⁺ T cells, naïve CD4⁺ T cells, and increased B and NK cell populations. Breast cancer patients had a lower levels of granulocyte ROS response compared to normal controls (p=0.0002). MBG treatment in vivo affected immune cell populations and responses studied ex vivo. Statistical model including all parameters across the whole trial showed an overall dose effect (P<0.01). In the high dose MBG group (10 mg/kg/day) subjects showed normalized response to PMA that was significantly higher than baseline (p=0.004). LPS stimulated CD14⁺ monocytes intracellular IL-10 production in the 6 mg/kg/day group subjects showed a significant increase over baseline after MBG treatment that was not seen in lower dose groups.

Summary: MBG enhanced antitumor effector cell immune response and IL-10 response in vitro but did not inhibit cytotoxic effects of chemotherapeutic agents. Our baseline studies of breast cancer patients showed that the patients have altered immune cell subsets and reduced responses compared to controls that were improved or restored in response to MBG. Statistical analysis showed significant dose effects on critical parameters of immune response.

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P17-10: MODULATING CYCLOOXYGENASE-2 MEDIATED PROSTAGLANDIN E2 PRODUCTION AND SIGNALING ON MURINE MAMMARY TUMORS ENHANCES NATURAL KILLER CELL LIGAND-RECEPTOR INTERACTIONS LEADING TO TUMOR CELL LYSIS AND DECREASED METASTASIS

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Breast malignancies often have high levels of cyclooxygenase-2 (Cox-2). The Cox-2 product prostaglandin E2 (PGE2) contributes to the high metastatic capacity of breast tumors. Our published data indicates that inhibiting either PGE2 production or PGE2-mediated signaling through the PGE2 receptors (EP1-4) reduces metastasis. To better understand the mechanism by which inhibiting PGE2 production or signaling reduces metastatic disease, we examined the role of Cox inhibitors and EP receptor antagonists in activating host immune defense. It is known that natural killer (NK) cell function is compromised by PGE2, but very little is known about the mechanism by which PGE2 affects NK effector activity. NK effector function is regulated by complex interactions of recognition receptors expressed on NK cells and cognate ligands

expressed on target cells. Both stimulatory and inhibitory signals are delivered through these receptor-ligand interactions. First, we showed that Cox inhibitors and antagonists of EP receptors modulate the expression of NK stimulatory (H60) and inhibitory (MHC class I) ligands in favor of enhanced NK recognition and killing of the tumor target cell. Masking of the H60 ligand on 66.1 mammary tumor cells inhibits NK mediated lysis showing the functional importance of H60. Inhibiting Cox-1 and Cox-2 on the murine mammary tumor cell line 66.1 with indomethacin increased expression of the activating ligand H60 and decreased the inhibitory MHC class I expression. As observed with Cox inhibitors, treating the tumor cell lines 66.1 and 410.4 with either EP2 or EP4 antagonists increases H60 expression and this is associated with an increase in NK-mediated lysis. Thus, blocking either PGE2 synthesis or signaling resulted in higher expression of the NK activating ligand H60. We then determined the direct effect of PGE2 on NK cell function. Our novel findings show that NK cells express all four EP receptors. Preliminary data show that the NK-EP receptor expression pattern changes during progressive tumor growth. We then asked whether these EP receptors could be classified as stimulatory or inhibitory receptors. Antagonism of EP receptors on NK cells alters cytolytic and migratory ability. These results together support a mechanism whereby inhibiting PGE2 production or signaling through EP receptors increases NK stimulatory ligand-receptor interactions and contributes to the mechanisms by which EP antagonists control breast cancer metastasis.

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P17-11: IDENTIFICATION OF NOVEL MIMOTOPES FOR THE DEVELOPMENT OF MULTIVALENT BREAST CANCER VACCINES

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Immunization against breast cancer antigens is a promising method to enhance current therapies since the immune system has the unique capacity to “seek-out-and-destroy” small deposits of tumor cells. Although clinical trials investigating breast cancer vaccines have generated promising initial data, it is clear that further refinement is required. We would like to develop multivalent vaccines that employ epitope libraries composed of shared antigens. We attempted to use a high-throughput method to screen and identify shared epitopes within breast cancer patients by serologic screening of random phage-display libraries. The advantage of the random peptides displayed by phage is that they can represent peptidomimetics of conformational and discontinuous antibody epitopes (“mimotopes”). Thus, this method is highly amenable to rapid identification of previously unknown epitopes that can be used directly as immunogens. Specifically, we used breast cancer patient serum to screen for breast cancer-specific mimotopes within a bacteriophage library presenting random cysteine-looped 12-mer peptides. The constrained nature of these cysteine-looped peptides promotes the peptides to adopt a tertiary structure that can mimic natural antibody epitopes, even though the mimotope may only share limited sequence similarity with the actual residues in the protein. In this way, we hoped to identify mimotopes specific for antibodies in breast cancer patient serum that could be used as the basis of a vaccine. In our initial screens, we only identified a limited number of specific phage. We subsequently determined that several of these “specific” phage were actually carrying mimotopes for Herceptin because some of the patients on our study were receiving Herceptin, a monoclonal antibody. To avoid isolation of phage specific for Herceptin in our screen, we developed high affinity Herceptin mimotopes that could be employed to remove Herceptin from the serum samples. Unfortunately, we failed to successfully employ the Herceptin mimotope in a manner that would remove Herceptin from our test serum. Future studies will require samples from patients who were not receiving monoclonal antibody therapy. Over the same period, we successfully refined our algorithms to enable epitope definition based on results from polyclonal antiserum by using a collection of 5 monoclonal antibodies with defined specificity as a test case. While this preliminary study supported the feasibility of this approach, we also identified several limitations that must be considered in future iterations of this screening method.

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P17-12: A NOVEL BREAST CANCER VACCINE USING IMMUNOSTIMULATORY PEPTIDES

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Background and Objectives: Immunotherapies have shown promising results in several cancers. One major challenge for immunotherapies is to break tolerance in order to achieve cytotoxic T lymphocyte (CTL)-mediated killing of tumor cells. For a successful induction of immune responses, dendritic cells (DCs), which are the most potent antigen-presenting cells, need to encounter both antigen and stimulus. If they

take up antigen without receiving a stimulus or at different times, they can induce tolerance. Therefore, it is crucial to avoid this scenario and maximize the number of DCs that encounter both antigen and stimulus at the same time to achieve an effective antitumor immune response. We will use nanoparticles that carry both antigen and stimulus to achieve this goal.

Our objective is to test if poly-lactic-glycolic acid nanoparticles (PLGA-NP) that are loaded with the HER2 peptide and carry the immuno-stimulatory peptide Hp91 on the outside or inside will allow antigen-presenting cells like DCs to induce the development of breast cancer-specific immune responses. We will also investigate to what extent this response will break T cell tolerance and prevent or delay the onset of breast cancer in HER-2/*neu* transgenic mice.

Methods: To conjugate peptide to the surface of the PLGA-NP, empty NPs were suspended in borate buffer by sonication for 30 sec. Zinc tetrafluoroborate hydrate was added to the NP suspension followed by a solution of Denacol® with stirring for 30 min at 37°C. The epoxy-activated NPs were separated by ultracentrifugation and washed with borate buffer to remove unreacted Denacol. For coupling to Hp91, the epoxy-activated NPs were suspended in borate buffer using sonication for 30 sec as above to which Hp91 dissolved in borate buffer was added. The reaction was carried out at 37°C for 2 h with stirring.

Results: We have designed PLGA-NPs that contain the immuno-stimulatory peptide Hp91 on the outside of the NP (PLGA-Hp91). Analysis of the NPs using atomic force microscopy suggests that the peptide was successfully conjugated to the NP surface. We tested whether conjugation of Hp91 to the surface of the NPs preserved the DC-stimulatory function of the peptide. Bone marrow-derived DCs (BM-DCs) from HER-2/*neu* transgenic mice were exposed to medium only or PLGA nanoparticles with Hp91 conjugated to the surface. After 48 h of culture, the DCs were analyzed by flow cytometry for expression of the DC maturation markers CD86 and MHC class II. We found that Hp91 when conjugated to the surface of PLGA-NPs induced increased expression of both CD86 and MHC class II similar to levels achieved with lipopoly-saccharide. Furthermore, to evaluate the toxicity of the PLGA-Hp91 NPs on BM-DCs, cells were either cultured in constant presence of the NPs for 48 h or exposed to NPs for 1 h at 37°C and then washed to remove the NPs. Both conditions produced similar maturation results, and no difference in viability was observed.

Conclusions: PLGA NPs that carry the immunostimulatory peptide Hp-91 on the surface are potent stimulators of mouse DCs and are nontoxic under the conditions tested. Thus, PLGA-Hp91 NPs are promising carriers for breast cancer-specific peptides and/or proteins since they will deliver the antigen cargo and provide the necessary maturation stimulus needed for proper T cell activation, which could lead to CTL-mediated killing of breast cancer cells.

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P17-13: A DNA VACCINE AGAINST FIBROBLAST ACTIVATION PROTEIN ACTS SYNERGISTICALLY WITH DOXORUBICIN TO INHIBIT TUMOR GROWTH AND METASTASIS IN A MOUSE MODEL OF BREAST CANCER

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The tumor stroma plays an important role during the growth and dissemination of tumor cells. Stromal cells produce growth factors, cytokines, and chemokines that activate adjacent extracellular matrix and induce the selection and expansion of neoplastic cells. Cancer-associated fibroblasts (CAFs) constitute the major cell type within the tumor stroma. In contrast to normal resting fibroblasts, the majority of CAFs have an activated phenotype; they synthesize factors such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β , and interleukin (IL)-10, which modulate tumor cell growth and contribute to the local chemosuppressive and immunosuppressive environment. CAFs also express cell surface markers unique to activated fibroblasts such as α -smooth-muscle actin and fibroblast activation protein (FAP). Our previous work demonstrated that administration of a DNA-based vaccine encoding the entire murine FAP gene delayed tumor growth in an orthotopic mouse model of breast cancer. Additionally, we demonstrated that the FAP vaccine in combination with doxorubicin therapy worked synergistically to significantly reduce or eradicate tumor growth. This effect was due in part to a reduction in collagen type I production resulting in increased uptake of doxorubicin in the primary tumors of vaccinated mice. In our present study, we have implemented the spontaneously metastasizing murine 4T1 breast cancer cell line to investigate the efficacy of our FAP vaccine in combination with doxorubicin therapy to prevent or inhibit metastatic growth. We predict that, in addition to preventing primary tumor growth, our FAP vaccine and doxorubicin combination therapy will inhibit the dissemination and/or establishment of spontaneously arising metastatic lesions. This approach may provide a new strategy to combat the major challenge of treating metastatic breast cancer disease.

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P17-14: IMMUNE RESPONSE AUGMENTATION IN METASTASIZED BREAST CANCER BY LOCALIZED THERAPY UTILIZING BIOCOMPATIBLE MAGNETIC FLUIDS

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Classical cancer therapy to reduce the tumor burden (radiation, chemotherapy) also significantly impacts the anti-tumor immune response by killing immune effector cells. Surgery results in removal of tumor burden so that the antigen pool is lost. We hypothesize that the immune response in disseminated breast cancer (BC) may be activated through the use of magneto-rheological fluids (MRF), suspensions of polarizable micron-size particles. MRF injected into a tumor solidifies under the influence of an external magnetic field, potentially initiating pro-inflammatory cytokine production resulting from the locally high stress on the neighboring tissue and tumor disruption due to attraction forces between the ferrous particles. Main objective is to design, characterize, and test MRF in biologically relevant models.

MRF is synthesized using magnetite particles and phosphate-buffered saline carrier fluids. Commercially available, superparamagnetic particles are conjugated with fluorescent dyes to provide imaging capabilities. The amine functionalized superparamagnetic particles were linked to an ester group from the fluorescent dye yielding a carboxamide. Fluorochrome-labeled superparamagnetic beads were co-injected with the MRF as a tracer for distribution studies in vivo. The distribution of the magnetic particles can be detected using fluorescent microscopy and flow cytometric analysis. A copolymer of N-isopropylacrylamide (NIPAAm) and N, N-methylene-bis-acrylamide (NBAAm), which forms a hydrogel with a lower critical solution temperature of 32°C, is used for biocompatibility. The monomers are polymerized on the surface of the iron particles using atom transfer radical polymerization. Additional work on methods development is currently focused on fluorescent label conjugation directly to iron particles.

Our team is developing a mathematical model to explore the effects of parameters such as particle size, concentration, magnetic field strength, and distance from magnet on the attraction forces between particles. These forces are hypothesized to result in the damage on the tumor cell lines and trigger the immune system response. Our studies will focus on minimizing the particle concentration and size required for an effective therapy by using a feasible magnetic field strength, less than 0.5 Tesla, at the targeted depth, about 2 mm from the surface.

The effect of MRF on primary and metastasized tumor growth will be evaluated by using an orthotopic murine BC model (4T1). Tumors will be evaluated by growth measurements and histological changes following injection of MRF or carrier fluid alone into the tumor and the effects of subsequent application of a magnetic field to the site. Effects on immune responses to the tumor, both primary and metastases will be assessed. Immunohistology and assessment of pro-inflammatory cytokine production will be determined.

This work is in progress and the results will be presented at the conference. It will establish the feasibility of augmenting immune responses to the tumor by mechanical disruption through MRF injection into the primary tumor and application of an external magnetic field. It is also expected to be directly relevant to metastatic disease by allowing for disseminated immune attack following initiation of local danger signals. Since the BC tumors are larger and closer to surface, the proposed method is easier to apply in a clinical setting.

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P17-15: DOES COMBINATION IMMUNOTHERAPY WITH HUMAN MONOCLONAL ANTIBODIES AGAINST HER2 AND CXCR4 AUGMENT BREAST CANCER CELL KILLING IN VITRO AND IN VIVO?

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The chemokine receptor CXCR4 and its ligand CXCL12 (SDF1 α) have been proposed to regulate the directional migration and invasion of breast cancer cells to sites of metastasis. Neutralizing the interactions of CXCL12/CXCR4 either by antibodies against CXCR4 or small-molecule antagonists impairs breast cancer metastasis in a mouse model. In addition, inhibiting CXCR4 with RNAi, or the specific antagonist, substantially delayed the growth of breast cancer cells in SCID mice. Therefore, the CXCR4 molecule could be a potential target to prevent breast cancer metastasis as well as growth. Human epidermal growth factor receptor-2 (HER2), which is overexpressed in about 30% of all breast cancers, has been a target for antibody-based therapy for advanced breast cancer. A humanized monoclonal antibody Herceptin® (trastuzumab) is currently in clinical use. Despite careful patient selection on the basis of ErbB2 expression, only a minority of patients responds to trastuzumab monotherapy. The linkage between CXCR4 and HER2, both of which play important roles in breast cancer progress, has been reported. HER2 upregulates the expression of CXCR4, which is required for HER2-mediated lung invasion and metastasis. There-

fore, we aimed to assess the antitumor effects of combinational immunotherapy by targeting both CXCR4 and HER2 in vitro and in a nude mouse breast cancer model. We have isolated human monoclonal anti-CXCR4 antibodies by phage display antibody library using paramagnetic proteoliposomes displayed CXCR4. Two extensively characterized antibodies were evaluated for effects on inhibition of proliferation on breast cancer cells in combination with Herceptin or alone. Their in vitro antiproliferation effects on MBA-MD-231-Her2+-CXCR4+ cells were equivalent to anti-Her2 Mab (Herceptin); no additive or synergistic inhibition of the combination was found. The two CXCR4 antibodies did not show a significant effect on prevention or treatment of tumor growth and lung metastasis of breast cancer in a nude mice breast cancer model, when used alone, as compared with a human isotype-matched control antibody. The combinational immunotherapy of the two anti-CXCR4 antibodies and Herceptin will be further tested in a nude mice model in the near future. Meanwhile, more anti-CXCR4 human antibodies with distinct epitopes than the two antibodies tested above will be identified in our continuous effort to test if combinational immunotherapy by targeting both CXCR4 and HER2 will be more effective than any single therapy.

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P17-16: IMMUNE MODULATORY EFFECT OF REGULATORY T CELLS ON NK CELLS AND THE DEVELOPMENT OF NOVEL THERAPEUTIC STRATEGIES AGAINST BREAST CANCER

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Natural killer (NK) cells protect against cancer by recognizing and lysing tumor cells and through the provision of cytokines such as IFN- γ to activate other effector cells. However, NK cells of breast cancer patients are often impaired. Cancer-mediated immune downregulation is attributed, in part, to regulatory T cells (Treg). Treg depletion using anti-CD25 monoclonal antibody (mAb) has been shown to improve T cell-based tumor clearance. While the suppressive effect of Treg on T cells is well documented, little is known about the effect of Treg on the homeostasis and function of NK cells. The main objective of this project was to investigate the modulatory role of

CD4+ CD25+ Treg on NK cells and to develop novel therapeutic strategies against breast cancer based on the combined depletion of Treg and activation of NK cells. In vitro, activated Treg (using anti-CD3 and anti-CD28 antibodies) potently suppressed NKG2D-mediated cytotoxicity of NK cells against tumor cells. The suppressive effect of Treg on NK cells was dependent on TGF- β but independent of IL-10 as determined using blocking antibodies. We assessed whether Treg influenced the homeostasis of NK cells in steady-state conditions. Treg were depleted from adult mice using an intraperitoneal injection of anti-CD25 mAb (clone PC61), and 4 days later NK cells were analyzed by flow cytometry or purified for functional assays. We observed that NK cells from Treg-depleted mice displayed a more mature phenotype compared to NK cells from control-treated mice as determined by CD11b, CD27, and KLRG1 expression. We further investigated the function of freshly isolated NK cells following Treg depletion. Purified NK cells from Treg-depleted mice displayed greater cytotoxicity against YAC-1 target cells and produced greater levels of IFN- γ upon IL-12 stimulation, consistent with our observations that Treg depletion enhances NK cell maturation. NK cells from transgenic mice expressing a dysfunctional dominant-negative TGF- β receptor also displayed a more mature phenotype, suggesting a role for both Treg and TGF- β in the homeostasis maturation of NK cells in steady-state conditions. We hypothesized that Treg depletion combined to NK cell activation with recombinant cytokines would induce potent antitumor effect. We tested the antitumor effect of Treg depletion combined to recombinant IL-21 administration against 4T1 mouse breast cancer. While Treg-depletion did not significantly affect 4T1 tumors tumor growth and administration of recombinant IL-21 slightly delayed tumor growth, Treg-depletion combined to recombinant IL-21 administration induced a synergistic antitumor effect, significantly delaying the growth of 4T1 breast tumors. We further tested the antimetastatic activity of Treg depletion in combination with recombinant IL-21 administration. Treg-depletion had little effect on the development of 4T1 lung and liver metastases while recombinant IL-21 significantly reduced the number of metastases in both the lungs and the liver. Notably, the antimetastatic effect of recombinant IL-21 was significantly enhanced when combined to Treg depletion. Taken together, our studies suggest that translational approaches combining a rational cocktail of monoclonal antibodies and cytokines to optimally coordinate innate and adaptive immunity may improve treatment of breast cancer.

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TARGETED THERAPIES I

Poster Session P18

P18-1: CHEMICAL REGULATION OF PROTEIN KINASES IN BREAST CANCER

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Small molecules that regulate kinase function have the ability to suppress signaling pathways that contribute to cancers. For example, the drugs Gleevec and Iressa, which target a small number of kinases are recent therapeutic advances that are used clinically for cancer treatments. In these situations, the goal is to inhibit oncogenic kinases that promote cancer. What about the other side of the equation? What about kinases and pathways that are naturally poised to inhibit disease? Our goal is to uncover these pathways and tap into their naturally protective potential for use as an innovative breast cancer therapy. This approach would seem to have validity because it would involve helping the body's own regulators block the initiation and development of cancer.

The problem with this approach is that most, if not all drugs, that bind to kinases behave as inhibitors. That was until recently, when Drs. Shokat and Walter at UCSF discovered the first drug that binds to a kinase and actually turns on another part of the proteins function. Indeed, this group discovered that ATP analogues directed at an inactive form of the kinase Irel were able to bypass its need for kinase activity and amazingly activate its downstream functions. This result has direct relevance to pseudokinases; the naturally occurring kinases that lack phosphorylation activity. Indeed, similarly to Irel, we hypothesize that pseudokinase domains function as nucleotide-dependent conformational switches, which through ligand binding serve to activate down stream functions. If true, drugs that target pseudokinases could behave as agonists, providing a completely novel mode of modulating signaling and cell behaviour. We aim to target pseudokinases, in a beneficial manner, for the treatment of breast cancer.

The pseudokinases that will be the focus of this effort are called the receptor guanylyl cyclases (RGCs). The RGCs regulate production of cGMP, a very potent intracellular chemical messenger. Recently, activation of RGC-A has been demonstrated to potently suppress growth and proliferation of breast adenocarcinomas. This function is dependent on elevated cGMP levels and may occur through cGMP-mediated inhibition of DNA synthesis leading to cell cycle arrest. A central hypothesis we seek to test is whether a drug, which increases RGC-A activity in breast cancer cells, can block DNA-synthesis and cause tumor cell death.

We will present our current efforts that are focused on investigating our ability to control the signaling functions of the RGCs and several other pseudokinases, including the JAKs and HER3. These efforts will clarify the general function of the pseudokinase domain and define its potential as a target for therapeutic intervention. Furthermore, we will present our efforts that are directed toward development of new chemical tools for investigating conformational dynamics in the protein kinase domain.

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P18-2: PRECISION-GUIDED CANCER THERAPY: SYNERGISM OF SELECTIVE TUMOR VASCULAR THROMBOSIS AND TUMOR MICROENVIRONMENT-ACTIVATED PRODRUG

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Targeted drug activation at selected sites promises reduced toxicity and enhanced efficacy. We have tested a bipartite drug delivery-activation system that employs selective tumor vascular-targeted induction of endogenous coagulation protease cascade followed by administration of a protease-activated prodrug. The efficacy of this strategy has been demonstrated by a fibronectin motif and tissue factor extracellular domain fusion protein that specifically activates coagulation on the tumor vascular endothelial surface when certain integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$, are expressed and exposed. The activation of the coagulation cascade and tumor vascular thrombosis as well as subsequent activation of thrombolytic pathways leads to the explosive amplification of serine protease cascades and local proteolytic activity within the tumor vasculature. This tumor-specific proteolytic activity has also been exploited for targeted local prodrug activation. The combination treatment of selective tumor vascular thrombosis and protease-activated prodrug demonstrated a profound synergism. Indeed, more robust and sustained tumor vascular thrombosis was observed compared to selective tumor vascular thrombosis alone. The activation of local tumor vascular thrombosis substantially increased prodrug activation and retention in tumors. Importantly, the activated prodrug eliminated the remaining tumor cells at the rim of tumors that do not depend on neo-angiogenesis for survival. This coordinated attack on tumors resulted in complete tumor eradication in multiple drug-resistant rodent and human tumor models with no apparent toxicity. The synergistic targeted activation of

coagulation and prodrug possesses interchangeable targeting potentials with different tumor vascular-specific molecules and thus may represent a general therapeutic strategy for breast cancer therapy.

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P18-3: INCREASED POTENCY OF THE PHSCN DENDRIMER AS AN INHIBITOR OF BREAST CANCER CELL INVASION AND SURVIVAL

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At the time of diagnosis, many breast cancer patients already have metastatic or locally advanced disease, and almost half of the patients diagnosed with early breast cancer develop metastatic or recurrent disease at a later time. Thus, metastasis is a very significant cause of mortality in breast cancer. Despite more than 30 years of research, metastatic breast cancer remains almost completely incurable; and after it is diagnosed the mean survival time for patients is only about 2 years. Thus, there is an urgent need for an effective, nontoxic systemic inhibitor of breast cancer cell invasion and survival. The $\alpha_5\beta_1$ integrin is a key receptor for both metastatic invasion and survival in breast cancer. The PHSCN peptide is a potent nontoxic inhibitor of serum-induced, $\alpha_5\beta_1$ -mediated invasion in vitro, and has proven to be an effective antitumorigenic and antimetastatic agent in vivo in animal models. It was also a very well-tolerated, systemic drug in a recent Phase 1 clinical trial and prevented disease progression for 4 to 14 months in approximately 40% of patients receiving it as a monotherapy. We made a significantly more potent derivative of the PHSCN peptide (Ac-PHSCN-NH2) by attaching it to a polylysine dendrimer with a short GGK linker sequence (Ac-PHSCN-GGK-dendrimer) and compared its potency with that of the PHSCN peptide as an inhibitor of $\alpha_5\beta_1$ -mediated, serum- or PHSRN-induced breast cancer cell invasion. We also compared the potencies of the PHSCN dendrimer and the PHSCN peptide as inducers of breast cancer cell apoptosis, as judged by the cleavage of PARP (Poly ADP-ribose polymerase), a known substrate of Caspase-3 and Caspase-7. We found that the PHSCN dendrimer is approximately 100-fold more potent than the PHSCN peptide at blocking serum-induced, breast cancer cell invasion (SUM 149 PT and SUM 52 PE). Since our published results have demonstrated that serum-induced invasion is mediated by the recognition of the PHSRN sequence by the $\alpha_5\beta_1$ integrin fibronectin receptor, we also compared the potencies of the PHSCN dendrimer and PHSCN peptide as inhibitors of PHSRN-induced invasion by serum-free SUM 149 PT and SUM 52 PE cells. We found that the PHSCN dendrimer was 100-fold more potent than the PHSCN peptide at blocking PHSRN-induced invasion. We have shown that FAK (focal adhesion kinase) phosphorylation on tyrosine 397 (P-Y397) is required for serum- or PHSRN-induced invasion; hence we compared the potencies of the PHSCN dendrimer and PHSCN peptide as inhibitors of serum-induced FAK P-Y397. Consistent with invasion inhibition results, we observed that the PHSCN dendrimer was about 1,000-fold more potent than the PHSCN peptide at blocking serum-induced P-Y397. Finally, we observed that the PHSCN dendrimer is at least 50-fold more potent than the PHSCN peptide at inducing PARP cleavage in SUM 149 PT cells, cultured in 5% serum. Since PARP is a known substrate of Caspase-3 and Caspase-7, these results suggest that the PHSCN dendrimer as a significantly more potent inducer of apoptosis in breast cancer cells, than is the PHSCN peptide.

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P18-4: A NOVEL AND EFFECTIVE COMBINATION TARGETING THERAPY FOR ADVANCED HUMAN BREAST TUMORS

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Mutations in tumor suppressor p53 facilitate tumor cell survival and resistance to chemotherapeutic drugs. Consequently, restoring p53 function within tumors is a promising strategy for targeted cancer therapy. Furthermore, vascular targeting agents for treatment of cancer are designed to cause selective shutdown of tumor blood vessels and offer yet another opportunity to reduce tumor load. Anionic phospholipids (AP), exposed on the surface of tumor endothelial cells, serve as an excellent marker for vascular disruption. Thus, treatment strategies that incorporate both of these pathways may result in improved and more potent responses. The aim of this study was to determine whether combination therapy targeting mtp53 and tumor blood vessels could be an effective therapeutic strategy for suppression of advanced breast cancer. We therefore tested therapeutic effects of PRIMA-1, which re-activates mtp53 and induces tumor cell apoptosis, and 2aG4, a monoclonal antibody that disrupts tumor vasculature by binding to AP on tumor endothelial cells, causing selective shutdown of tumor blood vessels. Two advanced breast tumor models that express mtp53 and are also Her-2/neu positive (BT-474) or negative (HCC-1428) were used to evaluate

this combination therapy. Fluorescence staining and tumor blood vessel perfusion assays were performed to determine therapeutic mechanisms of action. Our results showed that (1) combination treatment with PRIMA-1 and 2aG4 suppressed BT-474 tumor growth additively, leading to complete arrest of tumor progression during treatment; (2) some BT-474 tumors were completely eradicated when PRIMA-1 or 2aG4 was used alone, and combination treatment led to synergistic loss of tumors in nude mice; and (3) an increased antitumor effect was observed with PRIMA-1 plus 2aG4 treatment in HCC-1428 tumor model, and HCC-1428 tumors did not progress with 2aG4 or PRIMA-1 treatment and regressed slightly when combination treatment was tested; however, combination therapy did not lead to complete remission of HCC-1428 tumors; (4) the incidence of lymph node metastasis in nude mice bearing BT-474 breast tumors was reduced by combined treatment using PRIMA-1 plus 2aG4; and (5) no toxic effects were observed in any treatment groups. While seeking mechanistic explanations for antitumor effects we found that (1) PRIMA-1 induced exposure of AP in vitro in endothelial (HUEVC) cells, in BT-474, and extensively in HCC-1428 tumor cells and (2) PRIMA-1 plus 2aG4 treatment severely disrupted the ability of tumor blood vessels to perfuse in BT-474 and HCC-1428 tumors. These results indicate that PRIMA-1 plus 2aG4 combination therapy has a complementary and potent antitumor activity and could define a new strategy for suppression of advanced breast cancers.

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P18-5: TARGETING siRNA MISSILES TO HER2+ BREAST CANCER

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HER2+ breast tumors resist standard therapies, thus underscoring the need for alternative treatments. Our lab has previously developed novel non-viral vectors that target gene delivery to these cells. These vectors incorporate the specific receptor binding and endocytosis capacity of a human epidermal growth factor receptor (HER) ligand with the membrane penetration and intracellular translocation activity of recombinant adenovirus (Ad) capsid proteins. These recombinant proteins are highly efficient at receptor binding and cell penetration. However, delivering plasmid DNA requires additional DNA condensing agents that bind DNA well but release DNA poorly, thus hampering gene expression and complicating vector assembly.

As an alternative approach, we are exploring the possibility of using our targeted protein to deliver siRNA, which would simplify vector design and reduce the number of delivery barriers. Moreover, depending on the synthetic method, the siRNA may induce an interferon response that may contribute to tumor regression.

The purpose of this research is to test the hypothesis that recombinant Ad5 capsid proteins targeted to HER2+ breast cancer induce tumor cell-specific death through: receptor-targeted binding and cell entry; siRNA-mediated "knock-down" of specific gene transcripts; and cytokine-mediated cytotoxicity. The main objective of this study is to demonstrate that heregulin-directed proteins target siRNA delivery to HER2+ cells in vitro and in a xenograft mouse model of breast cancer.

Using confocal microscopy and ELISA, we found that high levels of the targeted carrier protein, HerPBK10, bind and enter HER2+ cells in culture while little to no protein bind HER2- cells. Using molecular weight filtration and gel electrophoresis, we confirmed that HerPBK10 and siRNA form noncovalent complexes spontaneously in vitro and remain stable under high speed centrifugation and filtration. Using an siRNA against HER2, we show by both immunoblotting and immunohistochemistry that the targeted complexes induce both HER2 and HER3 protein knock-down in HER2+ cells, while siRNA alone, carrier protein alone, or the same complex delivering a scrambled siRNA sequence had no effect on HER2 or HER3 levels. Untargeted lipoplexes delivering anti-HER2 siRNA had a small to modest effect on endogenous HER2. Testing targeted toxicity by metabolic assay showed that the targeted complex induced significant cell death to HER2+ cells while HER2- cells were little to modestly affected. A modest but significant level of IFN- α secretion was induced by the carrier protein and targeted complex in HER2+ but not HER2- cells, while untargeted lipoplex had similar negligible effect on both cell types. In vivo fluorescence imaging shows that the recombinant targeting ligand specifically accumulates in tumors and largely evades other tissues. In vivo targeting of the siRNA complex and testing targeted tumor cell death comprise our ongoing studies.

Altogether, we have found that the recombinant carrier protein, HerPBK10, can mediate targeted siRNA delivery to HER2+ cells and induce knock-down of target gene products, leading to cell death. Our in vivo studies examining the tumor-targeting ability of our recombinant ligand indicate that the carrier is effective at accumulating in HER2+ tumors.

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P18-6: MALONYL-CoA DECARBOXYLASE (MCD) INHIBITION IS SELECTIVELY CYTOTOXIC TO HUMAN BREAST CANCER CELLS

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Fatty acid synthase inhibition initiates selective apoptosis of human breast cancer cells both in vivo and in vitro that may involve malonyl-CoA metabolism. These findings have led us to explore malonyl-CoA decarboxylase (MCD) as a potential novel target for breast cancer treatment. MCD regulates the levels of cellular malonyl-CoA through the decarboxylation of malonyl-CoA to acetyl-CoA. While malonyl-CoA functions as a substrate for FAS, malonyl-CoA also inhibits fatty acid oxidation acting as a metabolic switch between anabolic fatty acid synthesis and catabolic fatty acid oxidation. In addition to its regulatory role, MCD prevents the accumulation of toxic levels of malonyl-CoA. We now report that treatment of human breast cancer (MCF7) cells with MCD small interference RNA (siRNA) reduces MCD expression and activity, reduces ATP levels, and is cytotoxic to MCF7 cells but not to human fibroblasts. Cytotoxicity is abrogated by pretreatment of MCF7 cells with TOFA (5-[tetradecyloxy]-2-furoic acid), an inhibitor of acetyl-CoA carboxylase that carboxylates acetyl-CoA to produce malonyl-CoA. In addition to siRNA to MCD, we synthesized a small molecule inhibitor of MCD, 5-[(Morpholine-4-carbonyl)-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-amino]-pentanoic acid methyl ester (MPA). Similar to MCD siRNA, MPA inhibits MCD activity in MCF7 cells, increases cellular malonyl-CoA levels, and is cytotoxic to MCF7 cells in vitro. Taken together, these data indicate that MCD-induced cytotoxicity is likely mediated through malonyl-CoA metabolism. These findings support the hypothesis that MCD is a potential therapeutic target for cancer therapy.

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P18-7: INTERACTIONS OF CYTOTOXIC SELENIUM-PROTEIN CONJUGATES WITH STANDARD CHEMOTHERAPY DRUGS: QUANTITATIVE ANALYSIS BY THE COMBINATION INDEX METHOD

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Conjugates of elemental selenium (Se) with certain proteins or lipoproteins represent a novel class of anti-cancer agents that has proven remarkably effective and selective in preclinical models, including multi-drug resistant mutant tumor cell lines. Their mechanism of action is only partially understood. The elemental Se component appears to be the cytotoxic entity and needs to be internalized by target cells in order to express its cytotoxic potential. Internalized Se-protein conjugates induce a rapid depletion of intracellular glutathione, the activation of several caspases, and a loss of plasma membrane asymmetry and mitochondrial potential. The (lipo)protein component has a dual role: it stabilizes the small particles of elemental Se and is largely responsible for the selectivity of cytotoxic conjugates, taking advantage of increased capacity of tumor cells to bind and internalize serum albumin and/or lipoproteins. Advanced breast cancer may be a particularly rewarding target for cytotoxic Se-albumin conjugates, as serum albumin constitutes about one fifth of the cytosolic protein content of clinical breast cancer specimens, and albumin content is inversely correlated with estrogen receptor expression. The objective of this study was to determine in a preclinical model how Se-protein conjugates interact with standard chemotherapeutic agents that are commonly used in the treatment of advanced breast cancer. MCF-7 breast cancer cells were exposed to graded doses of Se-protein conjugates, graded doses of standard drug, and various combinations of conjugates and standard drug for 1 hour. Surviving fractions were subsequently determined by in vitro clonal assay, and results were analyzed by the combination index method of Chou and Talalay (*Adv. Enzyme Reg.* 22: 27-55, 1984) to identify and quantify synergistic, additive, and antagonistic interactions. Combinations of Se-protein conjugates and doxorubicin showed a strong synergistic anti-tumor effect at all drug ratios tested. Combinations of Se-protein conjugates and 4-hydroperoxycyclophosphamide were nearly additive.

Combinations of Se-protein conjugates and cisplatin were mostly antagonistic, but the antagonism was less than expected based on previous pilot experiments with leukemia cells. Combinations of Se-protein conjugates and cisplatin were antagonistic most likely because cisplatin and elemental Se compete for the same binding site on serum albumin (cysteine-34). Analyses of additional drug combinations are in progress. So far, all data are consistent with the notion that cytotoxic Se-protein conjugates do not adversely interact with other anti-cancer drugs unless both agents compete for the same binding site on serum albumin.

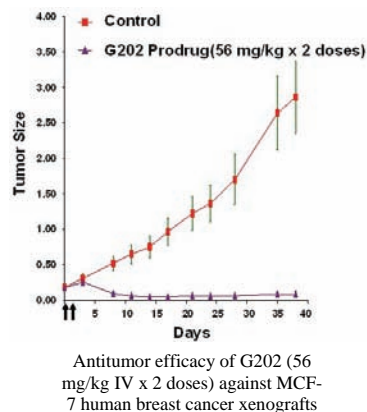
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0525.

P18-8: A PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) ACTIVATED THAPSIGARGIN PRODRUG DEMONSTRATES ANTITUMOR EFFICACY AGAINST HUMAN BREAST CANCER XENOGRAFTS

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Thapsigargin (TG) is a cytotoxic natural product isolated in high yield from the plant *Thapsia garganica*. In the NCI 60 Cancer Cell Line Screen, TG has a GI50 of $\sim 10^{-10}$ M which compares favorably with chemotherapeutic agents such as Paclitaxel (10^{-8} M) and Doxorubicin (10^{-7} M) in this assay. TG is a non-cell type specific cytotoxin that kills cells in a proliferation-independent manner via its potent inhibition of a critical intracellular protein, the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump. Sustained inhibition of the SERCA pump by TG produces an elevation of intracellular calcium to micromolar levels triggering multiple apoptotic pathways. Since TG has no therapeutic index in vivo (LD₅₀ in mice of 0.2 mg/kg), we generated prodrugs by coupling water soluble peptides to a potent TG analog, 12Aminododecanoyl-8-O deubutanoylthapsigargin (12ADT) to allow for systemic administration. In addition to solubilization, the peptide masks the cytotoxicity of 12ADT until it is released by specific active proteases present within tumor sites. In this study we coupled a series of acidic amino acids to the 12ADT analog to generate water soluble prodrugs. One prodrug (G202) was selected for further in vivo evaluation based on ability to be hydrolyzed by the carboxypeptidase PSMA. PSMA is expressed by prostate tissue with strongest expression in both primary and metastatic prostate cancers. PSMA, however, is also expressed by the neovasculature within most solid tumors including breast cancers but not by normal tissue vasculature. Pharmacokinetic studies demonstrated that a single dose of G202 at a dose of 56 mg/kg (i.e., ~ 150 -fold higher TG equivalents) produced a C_{max} of ~ 800 μ M and a plasma half-life of ~ 5 hr. Efficacy studies against PSMA-producing human prostate cancer xenografts demonstrated tumor regression and significant growth delay. G202 was particularly effective against breast cancer xenografts in which significant growth delay was observed following a single dose while $>90\%$ to complete tumor regressions were observed in the majority of treated animals following a single 2 dose course of G202. Biodistribution studies confirmed accumulation of the cleavage product Asp-12ADT in tumor tissues to micromolar levels, with 5–100-fold lower levels in other normal tissues sampled. G202 was stable to hydrolysis in blood with $< 0.5\%$ conversion observed over 24 hours. These preclinical results support the continued development and clinical testing of G202 as a novel anticancer agent for the treatment of advanced breast cancer.



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P18-9: USING COMBINATORIAL BORANE RNA CHEMISTRY TO CREATE NOVEL APTAMERS TO ErbB2

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Our laboratory has created the borane phosphonate moiety and has been dedicated to the understanding of the molecular consequences of these chemical analogues of DNA and RNA [2-7]. Borane ($-BH_3$) chemistry offers some unique chemical characteristics that make these compounds promising for enhancing the potential of two relatively new anticancer strategies: (a) the selection of tumor specific aptamers and (b) Boron Neutron Capture Therapy, a highly selective type of radiation therapy. Borane oligonucleotides are nuclease resistant and have increased lipophilicity compared to natural oligos, yet the modified borane nucleotide triphosphates (NTPaBs) still are efficiently recognized and utilized by RNA and DNA polymerase enzymes to enable selection of aptamers by the SELEX (Systematic Evolution of Ligands by EXponential enrichment) technique [6]. We hypothesize that borane-RNA aptamers will bind a

new diverse array of ligand sites in protein targets because of the distinct hydrophobicity, shape, and polarity properties imparted by the phosphorus-boron (P-B) chemical bond compared to the natural phosphorus-oxygen (P-O) bond. Further, the B-H group may form di-hydrogen bonds with an N-H group of the protein target, which is not possible with the natural P-O linkage [8]. These novel properties could lead to an increase in affinity and specificity, as well as stability of the nucleic acid to cellular nucleases. We have previously shown that borane modification dramatically enhances ss-siRNA (single stranded) and ds-siRNA (double stranded) function [2, 5]. RNAi has been shown to be effective in vivo through the delivery of small intracellular amounts of nucleic acid by targeting the cell surface with a cholesterol moiety [9]. It is likely that targeting a more specific cancer membrane target such as the ErbB2 receptor with a borane RNA aptamer may also be an effective two-prong strategy in breast cancer therapy. Like the antibody protein, Herceptin, an aptamer may assume a distinct shape and block the receptor. Further, borane aptamers can be used as specific carriers of α -P-borano-modified RNA aptamers against receptors highly over-expressed in breast and ovarian cancers should provide the opportunity of testing the efficacy of a target specific method for delivering boron to the cancer cells. Such boron molecules could be potent and unique anti-tumor surrogates for antibodies because of their ease in selection, smaller size, nuclease resistance, susceptibility to BNCT, and versatility in terms of adding chemical modifications to increase potency.

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P18-10: ORPHAN NUCLEAR RECEPTOR Nur77 AS A MOLECULAR TARGET FOR DEVELOPING Bcl-2-BASED THERAPEUTICS AGAINST BREAST CANCER

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Although anti-estrogen tamoxifen shows activities in preventing development of breast cancer, there are no effective therapies for malignant estrogen-independent breast cancer, which is also often resistant to chemotherapy and gamma-radiation therapy. Lack of such therapies has contributed to the high mortality for breast cancer. Novel therapies are therefore urgently needed. Recent progress suggests that elimination of cancer cells through induction of apoptosis is a plausible approach for developing new cancer therapies. Drugs that target the differences between tumor and normal cells may preferentially act on cancer cells but not normal cells, therefore reducing side effects or toxicity associated with administration of drugs, a major problem encountered by currently available therapies. Bcl-2, a potent inhibitor of cancer cell apoptosis, is overexpressed in majority of breast cancer and high levels of Bcl-2 are associated with resistance of breast cancer cells to a wide spectrum of chemotherapeutic agents and gamma-radiation therapy. Nur77, also known as TR3 or NGFI-B, is an immediate-early response gene, whose expression is rapidly induced by extracellular stimuli, such as growth factors, phorbol esters, and cAMP-dependent pathways, and apoptotic agents. Consistent with its induction by a variety of growth factors, Nur77 plays a role in cell proliferation and is often overexpressed in cancer cells. Paradoxically, Nur77 also functions as a potent pro-apoptotic protein, and its expression mediates apoptosis of cancer cells induced by certain chemotherapeutic agents. The apoptotic effect of Nur77 appears to be clinically relevant as downregulation of Nur77 is associated with metastasis of primary solid tumors, including breast cancer. Our laboratory recently discovered that the pro-apoptotic effect of Nur77 does not require its transactivation and DNA-binding activity. Instead, Nur77 translocates from the nucleus to mitochondria, where it targets mitochondria to trigger cytochrome c release. Nur77 targets mitochondria by binding to Bcl-2, a potent anti-apoptotic Bcl-2 family member. Importantly, the interaction between Nur77 and Bcl-2 induces a Bcl-2 conformational change, converting Bcl-2 from a cytoprotective to a cyto-destructive molecule. These results suggest a novel strategy for developing breast cancer therapeutics by targeting Bcl-2 conformation. We have identified a Nur77-derived Bcl-2-converting peptide (NuBCP-9) of only 9 amino acids length, which fully mimics the apoptotic function of Nur77. NuBCP-9 binds to Bcl-2, inducing a Bcl-2 conformational change and extensive apoptosis of breast cancer cells in vitro and in a mouse xenograft breast cancer model. The apoptotic effect of NuBCP-9 is not inhibited but rather potentiated by Bcl-2 overexpression. These properties distinguish NuBCP peptides from BH3 peptides and small molecules whose activities are attenuated by Bcl-2, identifying a new approach to target Bcl-2 for cancer treatment. Together, our results provide mechanistic insight into Bcl-2 conversion and identify a short peptide Bcl-2 converter and a new class of Bcl-2-based drug leads, representing a new direction for breast cancer drug development.

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P18-11: TARGETED DELIVERY OF 2-METHOXYESTRADIOL FOR THE TREATMENT OF BREAST CANCER

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Introduction: 2-Methoxyestradiol (2ME) has been shown to have both antiangiogenic and antiproliferative properties in various cell lines.^{1,2} It has also been shown to inhibit tumor growth in animal models.² However, clinical use of 2ME is limited by its short plasma half life and dose-limiting toxicity. In this project, our hypothesis is that by conjugating 2ME to water soluble N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers with targeting moiety attached to the side chains, it is possible to deliver the drug to the cancer cells. Our previous reports^{3,4} have indicated that HPMA copolymers containing the Arg-Gly-Asp (RGD) sequence attached to the side chains selectively accumulates in tumor tissue. Here we describe synthesis and characterization of HPMA copolymers containing varying amounts of RGDfK peptide attached to the side chains and the influence of RGDfK peptide content on binding affinity of the polymers. In addition, synthesis and characterization of HPMA copolymers containing 2ME and RGDfK are reported.

Experimental Methods: Comonomers of HPMA (MW 141.8), methacryloylglycylglycine-p-nitrophenyl ester (MAGGONp) (MW 321.7), methacryloylglycylphenylalanyleucylglycyl-2-methoxyestradiol (MAGFLG-2ME) (MW 801.4), copolymers containing varying amounts of RGDfK (1, 1.5, 3.5, and 12 RGDfK/polymer backbone) and a copolymer containing 2ME and RGDfK were synthesized by using similar strategies as previously described.^{3,4} All comonomers were characterized by NMR and mass spectrometry. Copolymer precursors and polymeric conjugates were characterized by size exclusion chromatography (SEC) for molecular weight, by amino acid analysis for RGDfK content and by fluorescence spectrometry for 2ME content. The bioactivity of copolymer-peptide conjugates was evaluated by adhesion inhibition of HUVECs on fibrinogen-coated plates as described previously.⁴ IC₅₀ values of copolymer-peptide conjugates and free RGDfK were determined by using a competitive binding assay with ¹²⁵I-Echistatin as described previously.⁵

Results: Free RGDfK and all polymeric conjugates of varying peptide content showed a significant ($p < 0.05$) degree of inhibition of endothelial cell adhesion to fibrinogen at 64 μ M (equivalent molar concentration of RGDfK). However no significant differences were observed in polymers and free RGDfK at all concentrations. Evaluation of IC₅₀ values for copolymers of varying peptide content revealed that highest affinity similar to free RGDfK was observed for the copolymer containing 12 RGDfK/polymer backbone. Lower affinity for copolymers containing lower RGDfK content may be attributed to limited receptor accessibility of each peptide on the polymer side chain. 2ME containing polymer had a M_w of 34,098 (n=1.7) and 2ME content was 1.4 mol %.

Conclusion: Our data suggest that binding affinity of HPMA copolymer-RGDfK conjugates is affected by peptide content. Binding affinity increases with higher peptide units per polymer chain. Currently work is under way to perform in vitro characterization of the synthesized drug-containing polymers for their chemical and enzymatic stability.

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P18-12: INHIBITION OF 6-PHOSPHOFRUCTO-2-KINASE DECREASES BREAST TUMOR GROWTH

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Phosphofructo-1-kinase, a rate-limiting enzyme of glycolysis, is activated in neoplastic cells by fructose-2,6-bisphosphate (Fru-2,6-BP), a product of four 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isozymes (PFKFB1-4). The inducible PFKFB3 isozyme is constitutively expressed by neoplastic cells and required for the high glycolytic rate and anchorage-independent growth of *ras*-transformed cells. siRNA silencing or small molecule inhibition (3PO) of PFKFB3 leads to a decrease in cell proliferation and soft agar colony growth of several breast cancer cell lines (MDA-MB-231, MCF-7, and MCF-10A-*ras*). Furthermore, 0.075mg/g 3PO administered via intraperitoneal injection suppresses tumor growth in both a MDA-MB-231 xenograft model (75% inhibition) and an activated *ras* (MMTV-*H-ras*) transgenic

breast cancer mouse model (30% inhibition). Taken together, these data support the clinical development of 3PO and other PFKFB3 inhibitors as chemotherapeutic agents against breast cancer.

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P18-13: DEVELOPMENT OF RADIO-TARGETING PEPTIDES FOR BREAST CANCER

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Breast cancer is the most common malignant tumor among women, accounting for an estimated 24% of all cancer cases. Despite the intensity of treatment with chemotherapy, radiation therapy, and surgery, breast cancer is the second lethal cancer for women, accounting for 18% of all cancer deaths. High-affinity and specific targeting ligands are strongly required to develop diagnostic and therapeutic agents for breast cancer. Small peptide-based agents have attracted wide interest as cancer targeting agents for the delivery of radionuclides or cytotoxic agents to tumors. These peptides are usually derived from binding motifs of known proteins or from phage-displayed peptide libraries with the limitation to L amino acids.¹ Here we report on the identification of peptidomimetics binding specifically to the breast cancer cells using "one-bead one-compound" (OBOC) combinatorial method.² The OBOC peptide libraries cXGXGXc with millions of permutations were synthesized by mix-split approach and screened using cell-growth-on-bead assay.³ Through sequencing the positive beads, we obtained maximal structure and activity relationship (SAR) information of potential targeting ligands for MDA-MB-231 cell line. For the identified peptide ligands LXY1 and LXY2 from the cXGXGXc OBOC libraries, anti- α 3 antibodies had obvious blocking effect using cell binding assays against different integrins, which strongly indicated that the peptide ligands are highly specific to α 3b1 integrin. In addition, the molecular interaction between the identified peptide ligands and α 3b1 integrin was characterized using α 3 alanine-scanning mutagenesis.⁴ Mutation of T162A, G163A, M164A, and T162F of α 3 inhibited α 3b1-mediated cell binding to the identified peptide beads. The binding site for the identified peptide ligands on α 3b1 was found to be close with laminin-5/ α 3b1 interaction. To identify the ligands with the highest affinity, we further constructed two highly focused OBOC peptidomimetic libraries basing on the SAR results and screened against MDA-MB-231 breast cancer cells. Furthermore, the binding affinities for the identified peptide ligands against MDA-MB-231 breast cancer cells were determined by flow cytometry analysis; LXY3 was found to show the high binding affinity to MDA-MB-231 breast cancer cell line (K_d=0.103 μ M, B_{max}=677). Additionally, this high affinity and specific biotinylated LXY3 was conjugated with optic probe streptavidine-Cy5.5 and successfully targeted to the MDA-MB-231 tumor in orthotopic xenograft nude mouse models. In conclusion, LXY3 is an excellent candidate for the development of a radio-targeting agent for breast cancer.

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P18-14: "SMART" POLYMERIC CARRIERS FOR ENHANCED INTRACELLULAR DELIVERY OF NUCLEIC ACID DRUGS

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Background: Upregulated expression of anti-apoptotic Bcl-2 and Bcl-xL proteins has been implicated in the development of breast cancer, increased malignancy, and resistance to standard chemotherapeutic agents. Inhibition of anti-apoptotic Bcl-2 and Bcl-xL protein expression using phosphorothioate antisense oligonucleotides (ASODN) appeared to be effective in increasing caspase-3-like protease activity and inducing apoptotic cell death in estrogen-sensitive and -insensitive breast cancer cell lines. However, in vivo therapeutic activity of ASODN is usually compromised by low serum stability, minimal and non-specific cellular uptake, and limited transport past the endosomal membrane, leading to diminished access to essential intracellular targets commonly present in the cytoplasm of cancer cells. The proposed research utilizes "smart" pH-sensitive membrane-destabilizing polymers to enhance the cytoplasmic delivery of anti-Bcl-2/Bcl-xL ASODN for treatment of breast cancer.

Objectives: (1) Synthesize novel “smart” polymeric carriers and evaluate their membrane-destabilizing activity in response to acidic pH gradients. Evaluate the intrinsic toxicity of new “smart” polymers on breast cancer cells. (2) Compare between polymer-ASODN complexes and free ASODN in terms of their ability to (a) suppress Bcl-2/Bcl-xL protein expression, (b) inhibit cancer cell growth, and (c) induce apoptosis in cancer cells. (3) Study the biodistribution of polymer-ASODN complexes upon intravenous administration into nude mice bearing tumor xenografts. Evaluate the in vivo activity of polymer-ASODN complexes in comparison to free ASODN as a function of their ability to (a) suppress Bcl-2/Bcl-xL protein expression, (b) induce apoptosis in tumor tissue, and (c) reduce tumor size.

Preliminary Results: We have designed and synthesized two classes of pH-sensitive membrane-destabilizing polymers. The first family of “smart” polymers is synthesized by free radical polymerization of pH-sensitive monomers (e.g., ethyl acrylic acid) and hydrophobic monomers (e.g., butyl methyl acrylate) forming the pH-sensitive membrane-destabilizing backbone. Cationic chains such as poly(trimethyl aminoethyl methacrylate) are grafted to the polymer backbone through acid sensitive linkages to complex therapeutic ASODN via electrostatic interactions. The second series is a family of star-shaped polymers where hydrophobic and cationic polymers are polymerized of the surface of a natural ring-shaped polymer namely β -cyclodextrin (β -CD). These hydrophobic and cationic grafts are anchored to the β -CD ring via acid-sensitive linkages, which degrade in response to endosomal pH-gradients. We expanded the number of polymers by varying the molar ratio of hydrophobic and pH-sensitive monomers and the molecular weight or the polymer backbone. The pH-dependent membrane-destabilizing activity of all polymers was evaluated using the red blood cells hemolysis assay, which identified several compositions that were hemolytic in response to acidic pH values (pH < 6.6). We evaluated the complexation of “smart” polymers with model ASODN using the gel retardation assay, which revealed the ability of these polymeric carriers to form stable complexes.

Significance: This research will provide a strategy to formulate DNA drugs into breast cancer therapies.

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P18-15: POLYAMINE ANALOGUES AS NOVEL ANTI-HER FAMILY AGENTS IN HUMAN BREAST CANCER

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The polyamines, spermine, spermidine, and putrescine, are naturally occurring aliphatic cations that are essential for normal cell growth and differentiation. A number of studies have shown that polyamines play a key role in carcinogenesis and malignant transformation, thus making the polyamine pathway a therapeutic target of interest. Increased levels of all three naturally occurring polyamines have been found in many types of cancers, including breast cancer. Polyamine analogues have been developed to mimic the three natural polyamines and exploit the self-regulatory properties of polyamines. Treatment of human breast cancer cell lines with polyamine analogues has been shown to inhibit cell growth and in some cases induce apoptosis. Polyamine analogues are divided into subsets based on their structure. One subset is conformationally restricted, long chain analogues named oligoamines. Our laboratory has focused on the oligoamine, CGC-11144, because of its effects in human breast cancer cells. Our studies have shown that oligoamines, especially CGC-11144, inhibit growth of human breast cancer cell lines in culture and in mouse xenograft models. Preliminary studies demonstrated the ability of CGC-11144 to downregulate two members of the human epidermal growth factor receptor (HER) family: epidermal growth factor receptor (EGFR/HER1) and human epidermal growth factor receptor-2 (HER2). The overexpression of EGFR and HER2 is usually associated with more aggressive tumors and worse prognosis. Preliminary studies have also shown that CGC-11144 inhibits cell growth in human breast cancer cell lines. Thus, the hypothesis underlying this proposal is that oligoamines are novel anti-HER family agents and oligoamine-induced down regulation of HER family members contributes to their cytotoxicity in human breast cancer cell lines. The objectives of this project are to investigate the mechanisms by which oligoamines downregulate EGFR and HER2 expression, determine the role of EGFR and HER2 in oligoamine-induced cytotoxicity, and determine if oligoamines can overcome endocrine resistance. Cell proliferation assays have demonstrated the ability of CGC-11144 and other oligoamines to inhibit cell growth in human breast cancer cell lines. These oligoamines can also suppress EGFR, HER2; and estrogen receptor(ER)-alpha protein in multiple human breast cancer cell lines. This suppression is both time and dose dependent. A relationship between oligoamine structure, growth inhibition, and suppression of EGFR and HER2 protein expression exists, with higher nitrogen compounds having the greatest effect. These results demonstrate that oligoamines are novel anti-HER family agents. The completion of this project will elucidate the role of oligoamine-induced down regulation of HER family members in human breast cancer cell lines' cytotoxicity and also provide valuable information about the potential clinical application of oligoamines.

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P18-16: TRAIL AND TRA-8 ANTI-DR5 ANTIBODY IN COMBINATION WITH CHEMOTHERAPY DRUGS PRODUCE INCREASED CYTOTOXICITY AND ACTIVATE APOPTOTIC PATHWAYS IN BREAST CANCER CELLS

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Background: TRAIL has been previously shown to induce apoptosis selectively in breast cancer cells but not normal cells. TRAIL binds to death receptors (DR4, DR5) and decoy receptors (DcR1, DcR2, OPG). TRA-8, an anti-DR5 agonistic antibody, inhibited breast tumor growth in xenograft models. Some breast cancer cell lines are resistant to TRA-8-induced apoptosis; however, Adriamycin[®] and Taxol[®] were shown to sensitize the cells in vitro in an additive or synergistic manner and inhibit breast tumor xenograft growth in combination with TRA-8. However, these studies did not address the mechanisms responsible for the increased efficacy of the combination treatment.

Objective: The central hypothesis is TRA-8 and chemotherapy can be combined to sensitize resistant breast cancer cells through enhancement of apoptosis and inhibition of antiapoptotic signaling. The objective is to identify mechanistic differences in response between cells of varying TRA-8 sensitivity to elucidate the molecular changes important in sensitization to death receptor therapy.

Methods: Cytotoxicity against human breast cancer cell lines was detected using an ATPase assay. Apoptotic proteins were identified by western blot analysis. JC-1 assays measured changes in mitochondrial membrane potential. siRNA methods were used to reduce XIAP expression.

Results: Examination of the TRAIL extrinsic apoptotic pathway showed that TRA-8 induced caspase-3, 8, and 9 cleavage in sensitive 2LMP and LCC6 breast cancer cells but not in resistant BT474 cells. TRA-8 combined with Adriamycin or Velcade generated caspase cleavage, PARP cleavage and reduced Bid in each cell line. Similar results were obtained when cells were treated with recombinant TRAIL, indicating TRA-8 activates the same mechanisms as the natural ligand. The anti-apoptotic protein, XIAP, was decreased in sensitive 2LMP and LCC6 breast cancer cells with TRA-8 treatment, but only combination TRA-8 and Adriamycin or Velcade treatment produced a decrease in XIAP in BT474-resistant cells. Pre-treating cells with a general caspase inhibitor blocked TRA-8-induced cytotoxicity and caspase cleavage but not Adriamycin cytotoxicity or the reduction of XIAP levels. Treatment with siRNA targeting XIAP increased BT474 cell line sensitivity to TRA-8 alone or in combination with Adriamycin. TRAIL and TRA-8 were shown to activate the intrinsic or mitochondrial apoptotic pathway by producing a loss in the mitochondrial membrane potential, which resulted in caspase-9 activation. Decreased mitochondrial membrane potential was detected in 2LMP and LCC6 breast cancer cell lines with TRA-8 alone and combination treatment; however, in the BT474 resistant cell line only combination treatment produced a reduction in membrane potential. In Adriamycin and combination-treated BT474 cells, Mcl-1, an antiapoptotic Bcl-2 protein that inhibits Bid, was reduced. These results indicate activation of the intrinsic apoptotic pathway with combination treatment.

Conclusions: TRA-8 or TRAIL induced cytotoxicity in sensitive breast cancer cell lines with activation of caspases and reduced expression of antiapoptotic proteins. However, only the combination treatments with chemotherapy produced these effects in resistant cells. Understanding the molecular mechanisms associated with breast cancer cell response to TRA-8 alone and with chemotherapy may lead to novel and superior therapeutic combinations for the treatment of breast cancer.

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P18-17: TARGETING MALIGNANT BREAST CANCER CELLS WITH A TUMOR-SPECIFIC NEUROTROPIC PEPTIDE-TOXIN

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Background and Objectives: During the initiation, development, and progression of breast cancer, certain receptors for cytokines or chemokines or neurotropic hormones may be upregulated in breast cancer cells but not expressed in normal mammary cells. It has been shown that greater than 50% of human breast cancer biopsy specimens exhibited binding sites for luteinizing hormone-releasing hormone (LHRH), a neurotropic peptide hormone secreted mostly from hypothalamus. The expression of specific LHRH receptors in a high percentage of breast cancers but not in most normal tissues (except brain) provides a rationale for the development of methods for therapy based on targeted cytotoxic LHRH agents. Pokeweed antiviral protein (PAP) is a 29-kDa plant-derived, potent ribosome-inactivating protein that inhibits protein translation and promotes cellular apoptosis. Since PAP does not contain the cell-binding domain, it cannot penetrate living cells and will not cause nonspecific cytotoxicity. The specificity and potent toxicity of PAP and the specific LHRH receptor expression

in breast cancer cells promote us to hypothesize that LHRH-PAP can specifically recognize and kill breast cancer cells and leave most normal cells unharmed. The objective of this proposal is to test a new concept of developing a breast tumor-specific neurotropic peptide-toxin and investigate the cytotoxic effect of LHRH-PAP on human breast cancer cells in vitro and the therapeutic efficacy and potential toxicity of LHRH-PAP in breast tumor animal models.

Brief Description of Methodologies: We plan to generate recombinant LHRH-PAP chimeric protein and examine the inhibitory effect of LHRH-PAP protein on tumorigenicity and tumor growth of breast cancer cells MDA-MB-231 and MCF-7 in cell culture. We will further determine the optimal dose of LHRH-PAP for treatment in vivo in orthotopic breast tumor mouse models, measure tumor sizes, record body weights (toxicity), and the mouse survival rates.

Results to Date: We have generated recombinant LHRH-PAPm fusion protein by constructing an expression vector containing maltose-binding protein (MBP) fused with LHRH-PAPm. After expressing the chimeric protein in *E. coli* in an inducible manner, we have purified LHRH-PAPm fusion protein by treatment with factor Xa and shown that the LHRH-PAPm fusion protein is relatively pure on SDS-polyacrylamide gel electrophoresis with a molecular mass 31 kDa. However, the yield of the fusion protein was low. We have also determined the cytotoxic effect of LHRH-PAP on human breast cancer cells MDA-MB-231 or MCF-7 in cell culture using cell survival and apoptosis assays. We showed that LHRH-PAPm fusion protein suppressed cell survival or proliferation of MDA-MB-231 or MCF-7 cells significantly in a dose-dependent manner. Our results suggest that LHRH-PAPm fusion protein exerts a significant cytotoxic effect on breast cancer cells. To purify a large quantity of LHRH-PAP fusion protein for animal studies, we have constructed a new expression construct in a pET-101 expression vector, and the purification of functional LHRH-PAP(H6) chimeric protein is under way.

Conclusions: We have been developing a breast tumor-specific LHRH-PAP toxin protein as a novel anticancer approach for breast cancer therapy. Our results indicate that this approach is effective in suppressing breast cancer growth. We will try our best to carry out the entire project successfully.

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P18-18: INDUCTION OF TRAIL BY CHEMOTHERAPY IN HUMAN BREAST CANCER CELLS

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The regulation of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in cancer chemotherapy is not fully understood. Here, we show that the HDACs (inhibitors of histone deacetylases) induce TRAIL in human breast cancer cells. Induction of TRAIL by the HDACi MS275 can be enhanced by adriamycin. We used reporter gene and chromatin immunoprecipitation assays to show that the transcription factor Sp1 is responsible for TRAIL induction by MS275 alone or in combination with adriamycin. Further, we find that the combined treatment of breast cancer cells with MS275 and adriamycin significantly increases apoptotic cell death via the activation of both death receptor and mitochondrial apoptotic pathways. Downregulation of TRAIL by small interfering RNA (siRNA) silencing decreased MS275-mediated adriamycin-induced caspase activation and apoptosis thus conferring adriamycin resistance. Importantly, breast cancer cell T47D in which Sp1 was knocked down or Sp1 knockout mouse embryonic stem cells were resistant to the combined treatments. Taken together, our results indicate that induction of TRAIL by the combined treatments with MS275 and adriamycin is mediated by Sp1 and suggest that transcription factor Sp1 is an important target for the development of novel anticancer agents.

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P18-19: INHIBITION OF LEGUMAIN:INTEGRIN COMPLEX AT INVASIVE CELL FRONT BLOCKS ANGIOGENESIS AND METASTASIS

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Previously we have demonstrated unique asparaginyl endopeptidases, legumain, is upregulated in a high percentage of solid tumors and plays an important role in tumor invasion and metastasis. To define the role of legumain in the molecular proteolytic pathways involved in cancer development, we examined legumain expression under hypoxia and found that legumain is induced by hypoxia and transported to the front of invading cells. Legumain forms a complex with integrins (such as $\alpha v\beta 3$ or $\alpha 5\beta 1$) on the cell surface of lamellipodia and invadopodia. In addition, binding of legumain to its cofactor integrin $\alpha v\beta 3$ significantly enhances its ability to activate pro-MMP2 and

pro-cathepsin L. Therefore, the legumain:integrin complex physically and functionally defines an invasive cell front surface (ICFS) where it regulates a network of matrix-processing enzymes. Legumain and multiple integrin subtypes are co-localized extensively on cell surfaces in immunohistochemical staining of MDA-MB-231 human mammary carcinoma cells. Inhibition of legumain:integrin by AEPI-1, a cell-impermeable legumain inhibitor, affects extracellular matrix remodeling and suppresses angiogenesis and cell invasion. Importantly, systemic administration of the AEPI-1 inhibits tumor angiogenesis and invasive growth. The treated tumors demonstrated profound disorganization of collagens and elastic fiber. This treatment prevented both spontaneous and experimental lung metastasis suggesting the activity of legumain:integrin is required for metastasis. The cell-impermeable asparaginyl endopeptidase inhibitor (AEPI-1) conjugated with FITC binds to legumain:integrin complex at the leading edge of the cancer cells indicating legumain:legumain complex is the target of AEPI-1. In addition, adenoviral transduction of legumain siRNA to MDA-MB-435 cells reduces the ability of this human breast cancer cell to metastasize to lung in immune-deficient mouse models. Our findings indicate legumain:integrin is an important regulator of cell invasion and matrix remodeling and an effective therapeutic target for cancer.

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P18-20: MAMMAGLOBIN IS PARTIALLY BOUND ON THE SURFACE OF BREAST CANCER CELLS

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Background and Objective: Mammaglobin (MAM) is homologous to the members of secretoglobins. MAM can be detected in the culture medium of breast cancer cells and the serum of breast cancer patients. MAM has been used as a serum marker for early breast cancer diagnosis. MAM protein consists of a small peptide with 93 amino acids in length. In a computer-assisted protein predictive analysis, a small fragment at the N-end was identified as a possible transmembrane domain. We propose that MAM partially stay associated with the surface membrane of breast cancer cells after secretion and the membranous-associated MAM may play essential roles in controlling cellular functions.

Methods: (a) Western blot hybridization was conducted to demonstrate the presence of membrane-bound MAM protein in human breast cancer cell lines that are known to be MAM positive (MDA-MB361 and MDA-MB415, ATCC); (b) Immunohistochemistry was performed on human breast cancer tissue sections to directly view the presence of membrane-bound MAM protein; (c) In vitro binding assay was carried out to test if the membrane-bound MAM can be specifically targeted by the fluorescent-tagged anti-MAM antibody; (d) To test if the membrane-bound MAM can be used as a molecular target for drug delivery, we further conjugated anti-MAM antibody to human LDL and loaded doxorubicin in the core of LDL particles. The anti-cancer effects of the anti-MAM conjugated, doxorubicin-loaded LDL particles were tested in vitro.

Results: Two MAM staining patterns were observed in 10 human breast cancer tissue sections. The majority of the MAM-positive cancer cells were shown as a cytoplasm positive pattern, as in previous reports by others. In some of the breast cancer cells, MAM-positive signals do appear at the membrane of the cancer cells. MDA-MB415 and MDA-MB361 cells were cultured, and the membrane and cytoplasmic proteins were isolated. Specific hybridization bands of MAM protein were detected via western blot assay in both protein compartments. The cancer cells were then incubated with the FITC-labeled anti-MAM, and clear fluorescent signals were observed on the surface of the cells. We further demonstrated that anti-MAM incubation activated PARP-1 protein in both cancer cells and caused cell apoptosis.

Doxorubicin (Dox) is commonly used for breast cancer therapy. We loaded this drug into the anti-MAM-labeled LDL (anti-MAM-LDL-Dox) and native LDL (LDL-Dox). The anti-cancer effects of these two therapeutic LDL particles were tested in vitro using MDA-MB415 and human aortic endothelial cells (HAEC). Both the cells were sensitive to free Dox. The LDL-Dox showed obvious cytotoxicity only on the HAEC cells, which are known to be LDL receptor positive while significant anti-cancer effect of the anti-MAM-LDL-Dox was only shown on MDA-MB415 cells.

Conclusion: We showed direct evidence that MAM proteins, at least some, attach to the surface of breast cancer cells and demonstrated that the specific binding of anti-MAM antibody to the membrane-bound MAM induces apoptotic death of breast cancer cells. MAM protein emerges as a promising molecular target for constructing targeted therapeutic tools.

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P18-21: HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN THE TREATMENT OF BREAST CANCER METASTASIS TO THE CENTRAL NERVOUS SYSTEM

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Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) are essential for neovascularization in cancer. VEGF expression is significantly correlated with microvessel density and the poor prognosis for a variety of human solid cancers including breast cancer. Therefore, new cancer therapies should be directed toward the tumor cells as well as the tumor vasculature.

Mesenchymal stem cells (MSCs) are derived from the bone marrow and were recently shown to serve as a vehicle for the delivery of anticancer agents into glioma tumors in mouse models. These cells have the remarkable capacity to migrate to tumors and damaged areas in the body in vivo. MSCs can be easily obtained from bone marrow, can be expanded in vitro, and are a prime target for tissue regeneration. These cells provide an important opportunity to express potent anti-cancer agents in the tumor microenvironment because of their contribution to the tumor stroma. MSCs can also be easily transduced and be gene-modified for therapeutic application. In this study, we examined the antitumor effects of genetically engineered MSCs expressing the antiangiogenic factor, sVEGFR-1. The idea is that MSCs engrafted in tumors may act as precursors for stromal cells and can serve as cellular vehicles for the delivery and local production of biologic agents.

First, we examined the potential of MSCs genetically engineered to express GFP to migrate to tumors formed in the mammary fat pads. Nude mice injected with MDA-MB-231 breast cancer cells formed a tumor after 2–3 weeks when transplanted into the mammary fat pads. We then injected MSCs/GFP cells i.v. and examined their potential accumulation and/or migration toward the mammary tumors in the fat pads. Unfortunately, we were not able to see migration of MSCs to these tumors. Future experiments will aim to inject MSCs/GFP directly to the tumor site and evaluate their effects on tumor inhibition. These studies are in progress.

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P18-22: NEW MOLECULAR INSIGHTS TO ACCELERATE THE DEVELOPMENT OF FATTY ACID SYNTHASE (FASN)-TARGETED INHIBITORS INTO A CLINICAL SETTING

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The difference in expression and activity of the lipogenic enzyme Fatty Acid Synthase (FASN) between normal and breast carcinoma cells provides an attractive approach to breast cancer therapy having the potential for a large therapeutic index. Indeed, studies with chemical inhibitors and, more recently, with RNAi-mediated downregulation of FASN gene expression have revealed that FASN inhibition has a dramatic impact on breast cancer cells. Breast cancer cells stop proliferating and ultimately die, at least in part, through the process of apoptosis. Unfortunately, few of the currently available FASN inhibitors (i.e., the mycotoxin cerulenin, the semi-synthetic compound C75 -*trans*-4-carboxy-5-octyl-3-methylenebutyrolactone-, the catechin epigallocatechin-3-gallate and other naturally occurring polyphenols, the beta-lactone Orlistat -tetrahydrolipstatin-, and the antibiotic triclosan -2,4,4-trichloro-2-hydroxy-diphenyl ether-) are expected to be “exclusively” selective for their FASN target, and their clinical relevance is limited as they exhibit pleiotropic effects due to chemical instability (cerulenin), undesirable side effects such as anorexia due to dual action as FASN inhibitors and *O*-carnitine palmitoyltransferase-1 (CPT-1) activators (C75), or poor solubility and extremely low oral bioavailability (Orlistat). Nevertheless, several cancer-associated molecular features, including nonfunctioning p53, overexpression of the Her-2/neu (erbB-2) oncogene, and hyperactivation of the PI-3’K downstream effector protein kinase B (AKT), appear to determine an exacerbated sensitivity to FASN inhibition-induced cancer cell death, and several reports from our group have described how FASN-catalyzed endogenous FA biogenesis also participates in the response of cancer cells to chemotherapeutic agents (e.g., anthracyclines such as doxorubicin, alkylating agents such as cisplatin, anti-metabolites such as 5-Fluorouracil, and microtubule-interfering agents such as docetaxel, vinorelbine, and paclitaxel), endocrine therapies (selective estrogen receptor modulators such as tamoxifen and faslodex), and HER1/HER2-targeting therapies (monoclonal antibodies such as the mono-HER2 inhibitor trastuzumab and tyrosine kinase inhibitors [TKIs] such as the mono-HER1 TKIs gefitinib and erlotinib and the dual HER1/HER2 TKI lapatinib). These findings should provide new avenues in the translation of existing FASN inhibitors as antitumor agents into a clinical setting. In this regard, it is hoped that the latest 4.5 Å resolution x-ray crystallographic map of mammalian FASN will lay a basis for efforts at structure-based drug design with this target, and therefore, the development of novel FASN inhibitors could advance more efficiently. We expect that the improvement in the selectivity and potency of forthcoming novel FASN-targeted small molecule inhibitors will direct the foundation of a new family of chemotherapeutic agents in breast cancer history.

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ANTIANGIOGENICS

Poster Session P19

P19-1: ANGIOTENSIN-(1-7) INHIBITS THE GROWTH OF HUMAN TRIPLE NEGATIVE BREAST TUMORS IN AN ORTHOTOPIC MODEL

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Triple negative breast tumors are an aggressive subtype of breast cancer that predominantly affects young African-American and Hispanic women with lower socioeconomic status. This form of breast cancer is correlated with a poor survival rate regardless of stage of diagnosis due to development of distant metastases. Triple negative tumors lack estrogen and progesterone receptors and have basal expression of the epidermal growth factor receptor-2 HER2 (ERBB2), severely limiting the therapeutic strategy for treatment. The purpose of this study was to determine whether angiotensin-(1-7) [Ang-(1-7)], an endogenous peptide hormone that activates the AT₍₁₋₇₎ receptor *mas*, can be used as a targeted chemotherapeutic agent for the treatment of triple negative breast cancer. Ang-(1-7) significantly inhibited the growth of MDA-MB-231 cells, a triple negative breast cancer cell line. More importantly, the heptapeptide significantly reduced the proliferation of human triple negative breast tumor growth in vivo, using an orthotopic model. Tumors resulting from the injection of human MDA-MB-231 cells in the mammary fat pads of athymic mice were treated for 28 days with either saline or 1000 µg/kg Ang-(1-7), delivered by subcutaneous injection every 12 h. The average volume of the tumors from mice treated with the heptapeptide was approximately 3-fold less than the size of the tumors from control animals (170.8±21.4 mm³; n=5, p<0.05 versus 546.7±87.9 mm³; n=5, p<0.05). In addition, Ang-(1-7) administration markedly reduced tumor weight, from 1.0±0.2 g in the saline-treated mice to 0.5±0.1 g in the Ang-(1-7)-treated mice (n=5, p<0.05). The decrease in tumor growth of Ang-(1-7)-treated mice was associated with a significant reduction in immunoreactive Ki67, a proliferation marker (from 84.2%±8.2 to 41.0%±7.6, p<0.05). The MAP kinase ERK2 also was reduced in the tumors from Ang-(1-7)-treated mice (from 2.0±0.5 to 0.7±0.2 densitometric units, p<0.05), in association with an increase in MAP kinase phosphatase DUSP1 mRNA (1.01±0.1 relative gene expression in tumors from saline-treated mice to 1.78±0.13 in tumors from Ang-(1-7)-treated mice), suggesting that Ang-(1-7) transcriptionally up-regulates DUSP1 to prevent MAP kinase activation and reduce cell proliferation and tumor growth. Tumors from mice treated with Ang-(1-7) also showed a decrease in the endothelial cell marker CD34 (87.8%±6.4 to 32%±7.0, p<0.05), demonstrating that the observed reduction in tumor growth was due in part to the inhibition of angiogenesis. Ang-(1-7) caused a dose-dependent reduction in endothelial tube formation in an in vitro Matrigel assay (maximal decrease of 54.3±5.8%). The anti-angiogenic effect of Ang-(1-7) was completely blocked by the specific Ang-(1-7) receptor antagonist [D-Pro⁷]-Ang-(1-7), suggesting that these effects are mediated by an Ang-(1-7) receptor. Ang-(1-7) also inhibited angiogenesis by more than 50% in vivo in the chorioallantoic membrane assay and the reduction was blocked by the AT₍₁₋₇₎ receptor antagonist. Based on the anti-proliferative and anti-angiogenic properties of the heptapeptide, Ang-(1-7) may be a new, first-in-class compound for the treatment of triple negative breast tumors targeting a specific AT₍₁₋₇₎ receptor *mas*.

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P19-2: BREAST CANCER THERAPY USING ANTIBODY-ENDOSTATIN FUSION PROTEINS

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The antiangiogenic protein endostatin has shown considerable antitumor activity in animal models. However, limited antitumor activity has been observed in human Phase I/II trials. Trastuzumab as a single agent has activity in HER2+ breast cancer when used alone or in combination with chemotherapy. Prior studies using an anti-HER2 antibody-murine endostatin fusion demonstrated enhanced antitumor effectiveness due to longer half-life, and selective targeting of endostatin that exceeded that of anti-HER2 antibody or endostatin given alone or in combination.

We have now constructed several anti-HER2 human endostatin fusion proteins by fusing human endostatin to the 3' end of a humanized anti-HER2 IgG3 antibody. Antibody targeting is designed to enhance local delivery of endostatin to tumor as well as increase endostatin half-life. In addition presentation of endostatin as a dimer due to fusion with each heavy chain may further augment activity. Two endostatin fusion proteins were generated using fusion with native human endostatin (huEndo) or with a mutant endostatin with a P125A substitution (huEndo-P125A) that has been reported to confer increased antiangiogenic activity. Native and huEndo-P125A fusion proteins inhibited endothelial vasculogenesis and proliferation of HUVEC in vitro and did so more efficiently than human endostatin. The huEndo-P125A fusion protein showed greater inhibition of vasculogenesis in vitro than either native endostatin or wild-type endostatin fusion protein. Treatment of established SKBR-3 xenografts in SCID mice with the anti-HER2 IgG3-huEndo-P125A (aHER2-huEndo-P125A) fusion resulted in complete regression and greater inhibition of growth, com-

pared to either anti-HER2 IgG3, human endostatin or the anti-HER2 IgG3-huEndo (aHER2-huEndo) fusion protein-treated mice. aHER2-huEndo-P125A also inhibited EMT6-HER2 tumor growth more effectively than PBS (*p* value = 0.003), huEndo (*p* value = 0.003), or aHER2-huEndo (*p* value = 0.004). aHER2-huEndo fusion proteins specifically targeted tumors expressing HER2 in syngeneic mice simultaneously implanted with murine mammary tumor cell line EMT6 and EMT6 tumor engineered to express the HER2 antigen (EMT6-HER2) on opposite flanks.

Linking endostatin to an antibody may significantly enhance antitumor activity of trastuzumab and endostatin. Mutant anti-HER2 huEndo-P125A fusion antibody showed markedly enhanced antitumor activity. Targeting antiangiogenic proteins using antibody is a versatile approach that could be applied to other tumor targets with alternative antibody specificities.

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P19-3: MICROBUBBLE AND ULTRASOUND ENHANCEMENT OF RADIATION-INDUCED TUMOR CELL DEATH IN VIVO

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Center for Medical Consumers

Objective: It is now appreciated that radiation not only damages the DNA inside tumor cells in vivo but may act by damaging the endothelial cells of the vasculature. In this study we test the hypothesis that microbubble agents in vivo may be used a priori to cause endothelial cell perturbations thus causing "radiosensitization" of tumors.

Methods: Human xenograft-bearing mice (48 animals) were exposed to ultrasound, ultrasound with microbubbles (1.67% and 3%), ultrasound with microbubbles with radiation (2 Gy and 8 Gy), and radiation alone (8 animals per group). For ultrasound treatments, animals were exposed to 16 cycles tone burst at 500 kHz center frequency and at 3 kHz pulse repetition frequency for 5 minutes. The peak negative pressure was set to 570 kPa (MI 0.8 at focus). For treatments involving bubbles, prior to ultrasound exposure Definity bubbles (Bristol Myers-Squibb) were administered at a nominal final iv concentration of 1.67%. For radiation treatments, animal tumors were exposed to 8 Gy of radiation (160 kVp x-rays). Tumors were assessed 24 hours after the administration of treatments by high-frequency spectroscopic ultrasound imaging for cell death detection and then removed after animal sacrifice for histological and immunohistochemistry-based analyses. Representative tumor sections were stained with haematoxylin and eosin stained and TUNEL stained for apoptotic cell death. Endothelial cells were labeled by CD31, and DAPI was used to label cellular nuclei. Tumors from two animals per condition will also be used for standard clonogenic assays. Assessment was carried out in a duplicate cohort of animals for tumor growth delay with daily measurements of size for up to 30 days after treatment.

Results: Analyses indicated an increase in tumor cell kill that increased when microbubbles were used in conjunction with radiation. Ultrasound treatment alone showed minimal cell perturbation and histological effect. Radiation alone induced sporadic patches of apoptotic cell death as before linked to vascular destruction. Ultrasound with microbubbles showed an induction of cell death within the focal zone. This effect significantly enhanced when treatment was combined with radiation doses. Histological patterns indicated significant vascular perturbation when the two modalities were combined. Estimates made from ultrasonic spectroscopy and correlative histopathology indicate a potential 4-fold enhancement of cell death when microbubble perturbations of tumor vasculature were combined with radiation. Such cell-death enhancement was limited to the volume of tumor where ultrasound was administered.

Conclusions: Radiation effects were enhanced by using microbubbles to perturb tumor vasculature prior to the administration of radiotherapy. This work forms the basis for ultrasound-induced spatial targeting of radiotherapy enhancement.

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P19-4: SYNERGISTIC EFFECT OF COMBINED NAB-PACLITAXEL AND ANTI-ANGIOGENIC ANTIBODIES IN INHIBITION OF TUMOR GROWTH AND METASTASIS IN BREAST CANCER XENOGRFT MODELS

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Southern Illinois University

Background: Many cytotoxic drugs show promise in clinical trials, but their beneficial effects are frequently not sustainable. This is mainly because unharmed and injured but alive tumor cells react to drug-induced necrosis and hypoxia by overproducing angiogenic factors. This is frequently followed by reactionary angiogenesis, leading to tumor regrowth and metastasis. We hypothesized that the effects of chemo-

therapy can be improved by combining chemo-drugs and antiangiogenic agents. To test this hypothesis, we used albumin-bound nanoparticle (*nab*-) paclitaxel (ABI-007 or Abraxane) that has already shown a superior efficacy against human metastatic breast cancer in clinics. As antiangiogenic complementary agents, we used anti-VEGF-A (bevacizumab, also known as Avastin) or vascular-targeting antiphosphatidylserine (PS) antibodies. The results of this study showed that combined therapy of *nab*-paclitaxel with Avastin resulted in synergistic inhibition of both primary large-sized breast tumors as well as lymphatic and lung metastases.

Methods: Luciferase-tagged MDA-MB-231-Luc⁺ human breast carcinoma cells were implanted into mammary fat pads of *nu/nu* or SCID mice and allowed to reach an average size of 450 mm³ (range 350–600 mm³). Mice were treated with Abraxane at 10 or 30 mg/kg on the qdx5 schedule for 1, 2, or 3 cycles with one rest week between the cycles. In combination groups, Avastin or anti-PS antibodies were administered at 4mg/kg) concurrently with Abraxane. Tumor growth was monitored for ~100 days after implantation. At the end of this period, normal organs were harvested and analyzed for luciferase activity representing metastatic spread.

Results: No toxicity was observed in any of the treated mice. Two cycles of high dose of Abraxane (30 mg/kg) combined with Avastin at 4 mg/kg resulted in complete tumor regressions in 100% of mice (n = 7) with large MDA-MB-231-Luc⁺ tumors. This was comparable to three cycles of Abraxane given at low dose of 10 mg/kg combined with Avastin that resulted in complete and sustainable regressions in 80% of mice (n = 5) and 98% reduction in the total metastatic burden in both lymph nodes and lungs. Combination with anti-PS antibody also inhibited tumor growth, but neither resulted in tumor eradication nor significantly affected lymphatic or pulmonary metastasis. In contrast, all cured mice by Abraxane and Avastin combination were free of metastasis as determined by measuring luciferase activity in lymph nodes and lungs.

Conclusions: Our data show that large-sized (up to 600 mm³) orthotopic tumors as well as lymphatic and systemic metastases can be eradicated using repetitive cycles of *nab*-paclitaxel at 30 mg/kg given concurrently with Avastin administered at 4 mg/kg. As an adjuvant therapy, Anti-VEGF-A antibody, Avastin, was superior in combination with *nab*-paclitaxel as compared with another potent anti-angiogenic agent, anti-PS antibody. The main reasons for curative potential of this combined therapy are likely favorable safety profile of Abraxane enabling delivery of high doses of cytotoxic drug without causing toxicity and the ability of Avastin to suppress treatment-induced reactionary angiogenesis.

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P19-5: TARGETING TISSUE FACTOR-EXPRESSING TUMOR ANGIOGENESIS AND TUMORS OF HUMAN BREAST CANCER XENOGRAFTS WITH EF24 CONJUGATED TO THE ACTIVE SITE-INACTIVATED FACTOR VIIa

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Tissue factor (TF) is aberrantly expressed on tumor vascular endothelial cells (VECs) and on cancer cells in many malignant tumors including breast cancer, but not on normal VECs and cells of benign tumors, making it a promising target for cancer therapy.

The angiogenic switch is turned on when microscopic tumors reach a millimeter size and begin secreting angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF induces TF on tumor-associated VECs. Hence, TF on the tumor-associated VECs should serve as a universal target. Since factor VIIa (fVIIa) is an endogenous ligand for TF, it is able to seek out TF-expressing microscopic metastases before they can be visualized by any conventional methods. When EF24 is conjugated to the active site-inactivated fVIIa, the conjugate will deliver it to those microscopic metastases and subsequently kill them.

Drug resistance is a major problem in cancer treatment. Emergence of resistance is due in part to genetic instability, heterogeneity, and high mutation rate of tumor cells. In contrast, endothelial cells are genetically stable, homogeneous, and rarely mutative. Therefore, anti-angiogenic therapy using conjugate directed against the TF-expressing endothelial cells associated with tumors should induce little or no drug resistance.

We synthesized a potent curcumin analog, EF24. We linked it to phenylalanine-phenylalanine-arginine-chloromethyl ketone (FFRck) and conjugated EF24-FFRck to fVIIa, a natural ligand for TF. The resulting EF24-FFRck-fVIIa conjugate binds to TF with an affinity similar to that of fVIIa. The TF-bound conjugate is endocytosed, carrying EF24 into the target cells. Our study showed that the EF24-FFRck-fVIIa conjugate inhibits angiogenesis significantly in rabbit corneas and Matrigels in mice. It also reduces human breast cancer xenografts in athymic nude mice. FFRck-fVIIa itself is a competitive inhibitor of fVIIa and is noncytotoxic so this drug carrier can prevent cancer patients from developing blood clotting.

Our conjugation procedure can easily be modified to replace EF24 with other drugs that are either more potent or have a different mechanism of action. This targeted drug delivery system may prove useful for treating drug-resistant tumors and micro-metastases that are clinically undetectable as well as primary tumors. The therapy by conjugating potent drugs to the fVIIa inhibitor has the potential to enhance therapeutic efficacy while reducing toxic side effects. The strategy also carries promise for treating other diseases caused by abnormal proliferation of blood vessels.

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P19-6: ANTIANGIOGENIC ACTION OF CHEMICALLY MODIFIED TETRACYCLINES IN BREAST CANCER

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The long term objective of this project has been to evaluate two nonantimicrobial chemically modified tetracyclines (CMTs), COL-300 and COL-308 (Collagenex Pharmaceuticals, Inc.), as inhibitors of the angiogenic response which maintains breast tumor growth and proliferation. COL-300 has shown efficacy in NCI-sponsored Phase I/II trials for reduction of the angioproliferative lesions in Kaposi's sarcoma. In our first EOH presentation of research sponsored by this award, we described effects of these two CMTs on release of Vascular Endothelial Growth Factor (VEGF) by two breast tumor cell lines, MCF-7 (which retains estrogen responsiveness and is not highly invasive) and MDA-MB-231 (estrogen insensitive and highly invasive), using an Enzyme-Linked Immunosorbent Assay (ELISA) for quantitation. We have now expanded our studies on release of VEGF from breast tumor cell lines to include two additional cell lines, MDA-MB-453 and MDA-MB-435s, which are more invasive than MCF-7. COL-300 stimulated release of VEGF from the cell lines except at the high dose of 50 μ M, but COL-308 inhibited release in a dose dependent fashion. At 20 μ M COL-308, a dose comparable to the COL-300 levels in the plasma of Kaposi's sarcoma patients, the release of VEGF from MCF-7 cells was suppressed virtually completely for up to 8 hours. TGF- β augmented VEGF release by the tumor cell lines, but this enhanced release was also inhibited by COL-308 in a dose-dependent fashion. The diminished VEGF levels in the presence of COL-308 could be ascribed to inhibition of synthesis and/or secretion, rather than degradation of pre-existing growth factor. Steady-state levels of the mRNAs for multiple VEGF species, measured by PCR, were unaffected by TGF- β or CMTs, whereas intracellular VEGF protein pools were diminished by COL-308, consistent with inhibition at the posttranscriptional level. Release of VEGF from the human monocytoid line Mono Mac 6, a model of tumor-infiltrating macrophages, was inhibited by >50% by 5 μ M COL-308 and by >90% by 20 μ M COL-308 in the absence of cytotoxicity. Intracellular VEGF pools in Mono Mac 6 cells were also diminished by COL-308, although by less than secretion; there was no significant effect of CMTs on the multiple VEGF mRNA species. Both COL-300 and COL-308 inhibited tube formation by human microvascular endothelial cells in a thick coating of Matrigel with IC50 values in the low μ M range and minimal cytotoxicity. Both CMTs also visibly diminished the morphogenic transition triggered by addition of type I collagen to endothelial cells, although by less than when cells were plated on Matrigel. Migration of endothelial cells through fibronectin-coated porous cell culture inserts was also inhibited by both CMTs. These results offer further support for the potential use of CMTs as antiangiogenic agents in management of breast cancer.

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P19-7: COPPER DEFICIENCY INDUCED BY TETRATHIOMOLYBDATE SUPPRESSES TUMOR GROWTH AND ANGIOGENESIS

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Copper plays an essential role in promoting angiogenesis. Tumors that become angiogenic acquire the ability to enter a phase of rapid growth and exhibit increased metastatic potential, the major cause of morbidity in cancer patients. We report that copper deficiency induced by tetrathiomolybdate (TM) significantly impairs tumor growth and angiogenesis in two animal models of breast cancer: an inflammatory breast cancer xenograft in nude mice and Her2/neu cancer-prone transgenic mice. In vitro, TM decreases the production of five pro-angiogenic mediators, VEGF, FGF2/bFGF, IL-1 α , IL-6 and IL-8. In addition, TM inhibits vessel network formation and suppresses NF κ B levels and transcriptional activity. Our study suggests that a major mechanism of the anti-angiogenic effect of copper deficiency induced by TM is suppression of NF κ B, contributing to a global inhibition of NF κ B-mediated transcription of pro-angiogenic factors.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0492.

P19-8: MEASUREMENT OF pO₂ AND pH WITH THREE-DIMENSIONAL RESOLUTION IN BREAST TUMOR MODELS IN LIVING MICE BY MULTIPHOTON MICROSCOPY DURING HERCEPTIN THERAPY

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Herceptin is emerging as a significant drug in the treatment of breast cancer and has become standard care for patients with HER2-positive cancer. Unfortunately, only a subset of patients benefits from therapy even with the addition of radiotherapy or chemotherapy. Hypoxia and acidosis are a hallmark of the metabolic microenvironment of breast tumors and hinder the efficacy of these treatments. We have shown previously in a xenograft model of breast cancer that Herceptin may “normalize” the tumor vasculature in a manner similar to that seen with antiangiogenic agents. The “normalization” of tumor vasculature would have profound implications on combined treatment with Herceptin by increasing oxygen and drug delivery to the tumor as well as increasing the pH. Increased pO₂ and pH in the microenvironment could improve breast cancer therapy with radiation and paclitaxel, respectively. These therapies would need to be administered at the appropriate time to optimize the period when the pO₂ and pH are at the most favorable levels otherwise there may be antagonizing effects between treatments. Unfortunately, there is little data on the effects of vascular “normalization” on the oxygen tension (pO₂) and acidity (pH) in the tumor microenvironment.

To address these outstanding questions, we have established a novel optical imaging technique using multiphoton laser scanning microscopy (MPLSM) to measure pO₂ and pH in breast tumors in vivo both during the progression of the disease and in response to therapy with Herceptin alone and combined with radiotherapy or paclitaxel.

Using MPLSM, high-resolution measurements of pO₂ and pH in the microenvironment of HER2-positive and -negative breast cancer xenografts implanted in the mammary fat pad (MFP) chamber model of transgenic mice were obtained. To measure pO₂, we modified the phosphorescence quenching microscopy technique for a two-photon excitation volume. To measure pH we used a novel fluorescent nanocrystal biosensor to perform ratiometric imaging. Initially, pO₂, pH and tumor vasculature morphology were noninvasively measured during the progression of the disease. Then the effect of Herceptin on the pO₂ and pH in the tumor microenvironment was examined with a focus on defining the “normalization” window induced by Herceptin. In all measurements, three-dimensional physiological maps of pO₂ and pH were made at multiple time points throughout the tumor. In all cases, tumor growth delay was charted while immunohistochemical analysis was performed to determine microvascular density and hypoxic fraction in select animals.

This protocol allows real-time tracking of dynamic changes in the pO₂ and pH of the breast tumor microenvironment during Herceptin treatment used with standard-of-care regimens. Our results may give further insight into the action of these treatments on breast cancer and provide information on how to rationally schedule combined therapy to provide for best possible outcome in patients with HER2-positive breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0436 and National Institutes of Health.

P19-9: TRANSCRIPTIONAL PROFILE OF VASCULAR TISSUE IDENTIFIES DISTINCT SUBTYPES OF VASCULATURE AND PREDICTS CLINICAL OUTCOME IN BREAST CANCER

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Background: Angiogenesis plays an important role in the progression of solid tumors, providing both nutrients required for growth and a way to escape the tumor bed. The level of vascularization, as measured by microvessel density, varies greatly between breast cancer patients. A high microvessel density significantly predicts poor survival in breast cancer, but between-study variation is high. It is also known that the tumor vasculature differs significantly from its normal counterpart. Among other changes, it is often leaky and generally less mature, lacking the functional pericytes that help stabilize the vessels. Exploitation of these differences has led to the development of several therapeutic avenues to target the tumor vasculature, most notably anti-vascular endothelial growth factor therapy. Several studies have helped characterize the gene expression of tumor endothelial cells in different cancers and identify tumor-specific endothelial markers. However, none had sufficient samples to investigate the variations that exist between patients.

Methods: To study endothelial gene expression, we performed microarray hybridization (N=32) of laser capture microdissected endothelial cells from invasive ductal carcinomas and matched endothelial morphologically normal adjacent tissue. We used various statistical techniques to analyze the data and compare it with the additional datasets from the published literature. Immunohistochemistry and polymerase chain reaction were used to validate the results.

Results: We identified two distinct subtypes of tumor endothelial cells in breast cancer patients. They are associated with tumors of high and low vascular density but not with recurrence. The gene expression and immunohistochemistry of pericyte markers offer evidence that the subtypes are also associated with vessel maturity. Surprisingly, most of the published markers of tumor endothelial cells are specifically associated with the low vascular density group. We also identified differences in the Notch and transforming growth factor-beta signaling pathways. Using the information from the subtypes, we developed a prognostic predictor of recurrence based on tumor vascular gene expression. Interestingly, this is independent of the microvessel density, identifying the recurrences in high and low density patients. The genes in this predictor are linked to several pathways linked to DNA repair, apoptosis, and energy production.

Relevance: This project will help us clarify our understanding of the process of vascularization in breast cancer and its role in tumor progression and metastasis. The identification of distinct tumor endothelial classes will help clarify the complex role that the vasculature plays in tumor progression. Our prognostic predictor reinforces that view and identifies differences in tumor vascular gene expression can be linked to distant recurrences. This differential expression points to pathways that could be involved in metastasis. This could lead to more individualized and potentially new treatment options.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0311; Genome Canada; Genome Quebec; Quebec Breast Cancer Foundation; Fonds de la recherche en sante du Quebec; Cedars Cancer Institute; and Genentech.

DRUG RESISTANCE I

Poster Session P20

P20-1: TAMOXIFEN-STIMULATED GROWTH OF BREAST CANCER DUE TO p21 LOSS

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Tamoxifen is widely used for the treatment of hormonally responsive breast cancers. However, some resistant breast cancers develop a growth proliferative response to this drug, as evidenced by tumor regression upon its withdrawal. To elucidate the molecular mediators of this paradox, tissue samples from a patient with tamoxifen-stimulated breast cancer were analyzed. These studies revealed that loss of the cyclin-dependent kinase inhibitor p21 was associated with a tamoxifen growth-inducing phenotype. Immortalized human breast epithelial cells with somatic deletion of the p21 gene were then generated and displayed a growth proliferative response to tamoxifen, whereas p21 wild-type cells demonstrated growth inhibition upon tamoxifen exposure. Mutational and biochemical analyses revealed that loss of p21's cyclin-dependent kinase inhibitory property results in hyperphosphorylation of estrogen receptor α with subsequent increased gene expression of estrogen receptor-regulated genes. These data reveal a new molecular mechanism of tamoxifen resistance and have potential clinical implications for the management of tamoxifen-resistant breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0325; Flight Attendant Medical Research Institute; Maryland Cigarette Restitution Fund; Susan G. Komen for the Cure; Elsa Pardee Foundation; Avon Foundation; and National Institutes of Health.

P20-2: THE UNFOLDED PROTEIN RESPONSE REGULATOR GRP78 AS A NOVEL TARGET FOR INCREASING SENSITIVITY OF HUMAN BREAST CANCER CELLS TO ANTIESTROGEN THERAPY

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Background and Objectives: Antiestrogen therapy is one of the most widely used and effective treatments for estrogen receptor-positive breast cancer. However, de novo or acquired resistance remains a major obstacle to patient survival. Understanding the molecular mechanisms responsible for resistance is critical for improving the treatment of breast cancer. It has recently been reported that estrogen starvation-induced apoptosis of breast cancer cells is mediated by BIK, an apoptotic BH-3-only protein located primarily at the endoplasmic reticulum. As a molecular chaperone in the endoplasmic reticulum, GRP78 exhibits cytoprotective functions due to its ability to maintain ER homeostasis, suppress stress-induced apoptosis, and control UPR signaling. GRP78 level is elevated in breast cancer and correlates with malignancy, metastasis, and chemotherapy resistance. The purpose of this study is to investigate the interaction between GRP78 and BIK, and the role of GRP78 in estrogen starvation-induced apoptosis in breast cancer cells.

Methods: Coimmunoprecipitation and GST-pulldown assays were used to probe the physical association between GRP78 and BIK. Adenoviral and plasmid expression vectors and siRNA were used to manipulate GRP78 and BIK levels. 293T cells and estrogen-dependent human breast cancer MCF-7/BUS cells were used as model systems. Cell apoptosis was examined by FACS analysis of BAX activation, mitochondrial membrane potential staining, and PARP cleavage quantitation. P³² labeling was used to evaluate the effect of GRP78 on BIK phosphorylation.

Results: We discovered that endogenous BIK selectively formed a complex with GRP78 probably through association with the cytosolic portion of ER transmembrane GRP78. In 293T cells, GRP78 bound to endoplasmic reticulum-targeted BIK and blocked its apoptotic activity but did not affect its phosphorylation status. In MCF-7/BUS breast cancer cells, overexpression of GRP78 inhibited estrogen starvation-induced BAX activation, mitochondrial permeability transition, and consequent apoptosis. Further, knockdown of endogenous GRP78 by siRNA sensitized MCF-7/BUS cells to estrogen starvation-induced apoptosis. This effect was substantially reduced when the expression of BIK was also reduced by siRNA.

Conclusion: Our results provide a new paradigm that an ER chaperone, GRP78, confers resistance to antiestrogen therapy in human breast cancer cells by inhibiting the apoptotic activity of BIK. Our studies suggest that GRP78, in addition to being a potential prognostic marker for responsiveness of human breast cancer to antiestrogen therapy, is a novel sensitizing target for such therapy. Combination of drugs suppressing GRP78 with conventional antiestrogen therapy may represent a novel approach to improve the effectiveness of and reduce the resistance to antiestrogen therapy.

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P20-3: INHIBITION OF JAB1 REVERSES RESISTANCE TO TRASTUZUMAB BY INCREASING THE STABILITY OF p27

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The development of the humanized monoclonal antibody, trastuzumab (Herceptin) targeting the HER2 (ErB2) receptor has provided promising treatment to patients with aggressive HER2 positive breast cancer. However, resistance is common with only 26% of HER2 overexpressing tumors responding to treatment and many tumors that do initially respond will regress within 1 year. Recent findings indicate that HER2 overexpressing breast carcinoma cells proliferate at a higher rate because of activated AKT phosphorylation of p27 resulting in cytoplasmic localization. Trastuzumab increases nuclear p27 protein levels by inhibiting AKT. Once in the nucleus, p27 inhibits Cdk2 activity and the cells remain in the G0/G1 phase of the cell cycle. Trastuzumab-resistant cells retain HER2 amplification and overexpression as well as inhibition of AKT when treated with trastuzumab. However, reduced nuclear p27 protein levels were observed in the resistant cells suggesting this may be a mechanism of resistance to trastuzumab. The Jun activation domain-binding protein 1 (JAB1) was previously found to negatively regulate p27 through nuclear to cytoplasmic relocalization and subsequent degradation. JAB1 overexpression has been observed in breast cancer and correlates with low p27 expression as well as poor prognosis. We examined whether JAB1 facilitated degradation of p27 may be one mechanism of resistance against trastuzumab treatment. Overexpression of JAB1 decreased p27 levels in a proteasome-dependent manner and increased the percentage of cells in S phase in the HER2-overexpressing breast cancer cell lines, BT-474 and SKBR-3. When SKBR-3 cells were treated with trastuzumab, p27 levels increased and G1 arrest was observed, but this was not seen when JAB1 was overexpressed. Increased JAB1 levels were observed in two BT-474 trastuzumab-resistant clones, C5 and C6. Targeted silencing of JAB1 increased p27 protein levels, reinstated a G1 checkpoint in tumor cells, and reduced cellular proliferation. We have observed that JAB1 overexpression is involved in breast tumorigenesis and overexpression protects against trastuzumab treatment by facilitating p27 degradation. Further, inhibition of JAB1 sensitizes resistant cells to treatment, thus inhibition of JAB1 is a novel strategy to sensitize tumors to trastuzumab-induced tumor growth arrest.

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P20-4: CONTRIBUTION OF AUTOPHAGY TO THE RESPONSE OF BREAST CANCER TO TRASTUZUMAB

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Trastuzumab (Herceptin[®]) is an effective therapy for a subset of breast cancer patients whose tumors over-express Her2, although many of these tumors fail to respond, while many others acquire resistance and recur. Continued administration of trastuzumab to resistant tumors can still reduce the rate of tumor growth; hence, patients can continue to benefit for a while. In vitro experiments may reflect this in vivo situation. Specifically, the growth of BT474 breast cancer cells is effectively suppressed by trastuzumab, but it is relatively ineffective at killing the cells. Trastuzumab inhibits the Her2 receptor and thereby decreases intracellular signaling through Akt. Activated Akt is well-recognized as a suppressor of apoptosis, yet in this model, cells survive the inactivation of Akt. Akt, through mTOR, is also a repressor of autophagy, a process by which a cell digests its own cytoplasm and organelles and recycles the constituents when energy supplies are low. Autophagy has been variously considered as an alternate mechanism of cell survival or cell death. Autophagy does eventually result in cell death, but for many days a cell is able to survive with the potential to recover between periods of drug administration. We hypothesized that growth suppression induced by trastuzumab would inhibit Akt and induce autophagy and that this would contribute to survival of tumor cells. Preliminary results have indeed demonstrated that trastuzumab induces autophagy in BT474 cells. Current experiments are directed to a comparison of the efficacy of trastuzumab in cells competent or incompetent to undergo autophagy. We hypothesize that inhibitors of autophagy may be combined with trastuzumab to enhance its therapeutic activity.

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P20-5: TARGETING GLUCOSYLCERAMIDE SYNTHASE REVERSES DRUG RESISTANCE THROUGH CERAMIDE-INDUCED APOPTOSIS IN VIVO

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Drug resistance in cancers blocks the cellular actions of chemotherapy and radiation therapy and then causes patients' failure in treatments. Characterizing a gene underlying drug resistance, as a novel therapeutic target, can significantly improve cancer treatment. Glucosylceramide synthase (GCS), a key enzyme catalyzing ceramide glycosylation in sphingolipid metabolism, is overexpressed in various drug-resistant cancer cell lines and in metastatic tumors. High GCS activity confers cell resistance by disrupting drug uptake and ceramide-induced apoptosis that is tightly associated with the efficiency of anthracycline, vinca alkaloids, and taxanes. To determine whether GCS is a target for the reversal of drug resistance, we evaluated the effects of antisense approach against GCS in vitro and in vivo. MBO-asGCS (mixed-backbone oligonucleotide, 20-mer) was designed to silence human GCS gene. MBO-asGCS (50 nM) substantially increased doxorubicin sensitivity in drug-resistant human (MCF-7-AdrR) and murine breast cancer cells (EMT6/AR1) by 83-fold and 43-fold, respectively. In contrast, MBO-asGCS only increased the doxorubicin cytotoxicity by two-fold in drug-sensitive cells and did not affect that cytotoxicity in human bone marrow mononuclear cells. This selective efficiency of MBO-asGCS for drug-resistant reversal is tied to silencing GCS overexpressed in drug-resistant cells. In the in vivo studies, we found that antisense approach against GCS significantly inhibited tumor growth and sensitized tumors to chemotherapy. Antisense GCS gene transfection dramatically inhibited orthotopic breast tumor formation in nude mice supplemented with estrogen since there was no tumor that appeared in mice inoculated with antisense-transfected cells (MCF-7-AdrR/asGCS) compared to 10 tumors in mice with drug-resistant parental cells (MCF-7-AdrR). The administration of MBO-asGCS (1 mg/kg/3-day, 42 days) inhibited tumor growth more than 64% (356 versus 983 mm³, N=10) and increased doxorubicin-sensitivity by 58% (187 versus 432 mm³, N=10) compared to MBO-scrambled alone and the combination of MBO-scrambled with doxorubicin (2 mg/kg/week), respectively. Further assessments of GCS gene expression levels, ceramide glycosylation, and apoptosis demonstrated that the effects of MBO-asGCS in vivo rely on the suppression of endogenous GCS overexpressed and the enhancement of ceramide-induced apoptosis. These evidences pinpoint that GCS is a novel target for the reversal of drug resistance in cancers.

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P20-6: TARGETING THE STABILITY OF p27KIP1 CELL CYCLE INHIBITOR WITH SMALL MOLECULES

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Metastasis is the final step in the progression of breast cancer. Currently available therapeutic strategies at this stage of cancer progression are often nonspecific, of low effectiveness, and highly toxic. Therefore, new therapeutic strategies are required, exploiting novel targets. JAB1/CSN5 protein is a negative regulator of p27 cell cycle inhibitor that holds considerable promise as a therapeutic target for breast cancer. A loss of the p27 protein is found in 50% of breast cancer cases and contributes to the uncontrolled proliferation of malignant cells because p27 is a negative regulator of the protein kinases Cdk2/cyc E, which drive cells to the S phase of the cell cycle. JAB1 overexpression has been implicated in the pathogenesis of several types of human cancer via its negative control of mammalian cell-cycle checkpoints by inactivating p27. This inactivation results from the sequestration of p27 in the cytoplasm, where it is degraded resulting in accelerated cell cycle progression. Our early studies have demonstrated that JAB1 protein expression in breast cancer inversely correlates with p27. Moreover, patients whose tumors did not express JAB1 had the highest survival rate, while those with high JAB1 levels had a poor prognosis. When we overexpressed JAB1, it synergistically increased cell proliferation, disrupted cell shape, and adhesion, and increased cellular invasion. Furthermore, JAB1 was found to be one of the most significant genes within a gene-signature set that is correlated with the propensity of human breast cancer cells to metastasize. These alterations are all characteristic of metastatic cancers. On the basis of these data, we hypothesize that JAB1's oncogenic function is an ideal target for breast cancer therapy. However, new strategies need to be developed to block JAB1's oncogenic function, because silenced JAB1 with shRNA that was used in xenograft models cannot be applied to human therapy. We propose that the best opportunity to control JAB1 activity will be to use small molecules that interfere with the interaction between JAB1 and p27. We have developed an innovative approach to block the degradation of p27 by targeting JAB1. Our objective will be to screen a small-molecule drug library to identify molecules that specifically disrupt the interaction between JAB1 and p27. Our second objective

will be to validate the inhibition of the JAB1-p27 interaction using in vitro and in vivo assays. Disruption of JAB1's oncogenic activity may be a highly effective and safe treatment for patients with advanced breast cancer, given the clear relationship between high JAB1 expression levels and aggressive breast cancer cell behaviors. JAB1 has only very recently been discovered as a key gene during cancer progression and is not yet being developed as a therapeutic target.

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P20-7: INTERMITTENT TREATMENT STRATEGIES USING AROMATASE INHIBITORS: A PRECLINICAL STUDY

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Three third-generation aromatase inhibitors (AIs), anastrozole (ANA), exemestane (EXE), and letrozole (LET), are important drugs for hormonal therapy of breast cancer in postmenopausal women. Currently, a major setback with using these drugs is the development of acquired resistance. In addition, we do not have a definitive answer as to whether a non-cross resistance between steroidal AI (i.e., EXE) and nonsteroidal AIs (i.e., ANA and LET) exists. With such considerations, our laboratory has initiated a preclinical research project that we believe will be valuable for the design of future clinical trials on AIs. MCF-7aro, an ER+ and aromatase overexpressing cell line, was used to generate a series of AI-resistant lines. Results generated so far from our laboratory indicate that mechanisms of inhibition and resistance of EXE are different from those of ANA and LET. Based on these findings, experiments have been carried out to test the hypothesis that intermittent use of a single AI, that is EXE will decrease/delay acquired resistance compared to conventional therapies. Three types of experiments have been performed:

1. MCF-7aro cells were cultured in the presence of T and an intermittent use of a single AI (e.g., 1 week on and 1 week off, 2 weeks on and 1 week off). The time to progression (resistance) for each condition was compared to that attained with continuous treatment of the AI. Results generated have revealed that resistance developed after continuous treatment of EXE for 8–10 weeks. On the other hand, acquired resistance was developed in the three intermittent groups after treatment for 32 weeks.
2. EXE-resistant MCF-7aro cells developed after continuous treatment were further treated with EXE in an intermittent manner. The cells initially responded to the intermittent treatment and became resistant after 8 weeks.
3. ANA-resistant MCF-7aro cells were treated with EXE intermittently. While these experiments are still ongoing, we have learned that ANA-resistant cells respond to EXE at least up to 6 weeks. We also compared the effects of continuous and intermittent use of a nonsteroidal AI, that is, LET. The time to progression (resistance) was found to be identical for the two culture conditions for LET.

Therefore, we have not observed any advantage for an intermittent use of LET. We believe that these results will serve as an important basis for the design of intermittent therapies using AIs such as EXE. While one may argue that results generated from such cell culture experiments cannot be immediately translated to clinical applications, the reality is that not enough valid information is available for the design of new clinical trials. The results generated from our proposed study will serve as an important basis to improve the efficacy of AIs in the treatment of hormone-dependent breast cancer.

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P20-8: CHROMOSOME 8q22 AMPLIFICATION CONTAINS LAPT4B, IMPLICATED IN RESISTANCE TO CHEMOTHERAPY IN BREAST CANCERS

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Background: Chemotherapy is applied to eradicate residual tumor cells outside the breast after surgical removal of primary tumors. Residual tumor cells resistant to chemotherapy have a survival advantage in treated patients and progress to metastatic disease. We recently mapped a copy number gain at 8q22 in primary breast tumors and found this genetic feature was associated with distant metastatic recurrence in patients who had received post-operative anthracycline-based chemotherapy. Lysosomal-associated protein transmembrane-4 beta (LAPT4B) is located at 8q22. Like its family member LAPT4A, it has the same conserved YXXF and VL motifs for lysosome and/or endosome targeting. Human LAPT4A expressed in drug-sensitive yeast decreased cell sensitivity to various compounds, including anthracyclines routinely used for chemotherapy in breast cancers. However, LAPT4A is not overexpressed in human cancer. We hypothesize that the related protein, LAPT4B, may be a putative drug resistance gene relevant to chemo-sensitivity in breast cancers.

Methods: Breast tumors (n=115) from patients with at least 3 years of clinical follow-up, and who received post-operative chemotherapy, were arrayed on Affymetrix U133 expression arrays. DNA copy number was analyzed in a subset of these tumors using Affymetrix 10K SNP arrays. Fifteen breast cancer cell lines were examined for LPTM4B expression by RT-real-time PCR and for drug sensitivity by evaluation of IC₅₀ values in a cell viability assay. Three RNA interference (RNAi) oligos targeting LPTM4B were used for knockdown experiments. Auto-fluorescence for daunorubicin was examined to determine drug intracellular localization.

Results: LPTM4B transcripts levels were significantly higher in high-grade compared to low-grade breast tumors and normal breast tissues. Over-expression was correlated to 8q22 DNA copy gain and occurred more frequently in tumors with distant recurrence than those without recurrence (p=0.0019). Among breast cancer cell lines, LPTM4B transcripts levels varied and showed a significant correlation with IC₅₀ for anthracyclines (R² = 0.45), but not to taxol and cisplatin. LPTM4B knockdown with either of three RNAi decreased transcripts levels by 80-90%. Its knockdown increased sensitivity to anthracyclines by two to four-fold in five of six breast cancer cell lines that originally expressed high level LPTM4B. This increase in sensitivity was associated with an acceleration of the nuclear localization of the anthracycline, daunorubicin. Furthermore, RNAi knockdown of LPTM4B significantly suppressed cell growth in three of the six cell lines.

Conclusion: LPTM4B expression contributes to resistance to anthracyclines in breast cancer cells through the sequestration of anthracyclines into cytoplasmic organelles and away from nuclear DNA target. This effect may partially account for survival and recurrence of residue tumor cells with 8q22 gain/amplification after anthracycline-based post-surgical chemotherapy. LPTM4B represents a new predictive marker for drug selection and a new target for enhancing efficacy of chemotherapy against breast cancers.

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P20-9: Akt PLAYS AN IMPORTANT ROLE IN BREAST CANCER CELL CHEMOTAXIS TO CXCL12

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The chemokine receptor CXCR4 is functionally expressed on the cell surface of various cancer cells and plays a role in cell proliferation and migration of these cells. Specifically, in breast cancer cells, the CXCR4/CXCL12 axis has been implicated in cell migration in vitro and in metastasis in vivo, but the underlying signaling mechanisms are incompletely understood. The xenograft-derived MDA-MB-231 breast cancer cell line (subline 231mfp), which was shown previously to grow more aggressively than the parent cells, showed increased CXCR4 expression at the mRNA, total protein and cell surface expression level. Interestingly, CXCR4 expression was not limited to the cell surface but was quite prominent in the nucleus. The increased CXCR4 expression in 231mfp cells correlated with an enhanced response to CXCL12/SDF-1α, specifically in augmented and prolonged Akt activation in a G(i), Src family kinase and PI-3 kinase-dependent fashion. 231mfp cells migrated toward CXCL12 in contrast to the parent cell line and this chemotactic response was blocked by inhibition of G(i), Src family kinases, PI-3 kinase and interestingly, Akt itself, as could be shown with two pharmacological inhibitors, a dominant negative Akt construct and with Akt shRNA. Collectively, we have demonstrated that prolonged Akt activation is an important signaling pathway for breast cancer cells expressing CXCR4 and is necessary for CXCL12-dependent cell migration.

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P20-10: ALTERATIONS OF MICRORNAS EXPRESSION ARE ASSOCIATED TO MULTIDRUG RESISTANCE OF BREAST CANCER

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Background: Multidrug resistance (MDR) has been frequently associated with elevated expression of one or more ATP binding cassette (ABC) transporters such as three well-known drug efflux proteins: P-glycoprotein (MDR-1), multidrug resistance associated protein (MRP-1), and breast cancer resistance protein (BCRP). However, the regulation of these transporters remains controversial. A recently discovered class of small, functional, noncoding RNAs named microRNAs (miRNAs), has been shown to function as regulatory molecules by inhibiting protein translation and to play an important role in development, differentiation, cell proliferation, and apoptosis. Very little is currently known if miRNAs affect MDR.

Purpose: Our purpose is to find a potential novel mechanism involved in MDR and a cluster of miRNAs that may directly serve as potential drugs for MDR reversal. We

will also identify a specific expressive pattern of miRNAs in MDR cells that may be used to diagnose MDR in breast cancer patient samples.

Results: In this study, we performed analyses of miRNA expression in MDR cell lines compared to their parental cell line using a miRNA microarray. Specific miRNA expression profiles in MDR cells were observed. The alteration of expression of 115 miRNAs were found at least in one MDR subline, 18 of which were differentially expressed in all three MDR sublines compared to their parental cells. miR-326 has demonstrated the involvement in multidrug resistance. Our findings will be beneficial for the prediction of MDR in patients as well as the design of personalized therapy for breast cancer patients.

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P20-11: MACROAUTOPHAGY INHIBITION SENSITIZES TAMOXIFEN-RESISTANT BREAST CANCER CELLS AND ENHANCES MITOCHONDRIAL DEPOLARIZATION

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Macroautophagy (autophagy), a process for lysosomal degradation of organelles and long-lived proteins, has been linked to various pathologies including cancer and to the cellular response to anticancer therapies. In the human estrogen receptor-positive MCF7 breast adenocarcinoma cell line, treatment with the endocrine therapeutic tamoxifen was shown previously to induce cell cycle arrest, cell death, and autophagy. To investigate specifically the role of autophagy in tamoxifen-treated breast cancer cell lines, we used a siRNA approach, targeting three different autophagy genes, *Atg5*, *Beclin-1*, and *Atg7*. We found that knockdown of autophagy, in combination with tamoxifen in MCF7 cells, results in decreased cell viability concomitant with increased mitochondrial-mediated apoptosis. The combination of autophagy knockdown and tamoxifen treatment similarly resulted in reduced cell viability in the breast cancer cell lines, estrogen receptor positive T-47D and tamoxifen-resistant MCF7-HER2. Together, these results indicate that autophagy has a primary pro-survival role following tamoxifen treatment and suggest that autophagy knockdown may be useful in a combination therapy setting to sensitize breast cancer cells, including tamoxifen-resistant breast cancer cells, to tamoxifen therapy.

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P20-12: INHIBITION OF GLYCOLIPID METABOLISM DOWNREGULATES MDRI EXPRESSION IN ADRIAMYCIN-RESISTANT CANCER CELLS

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The development of resistance to chemotherapy represents a formidable barrier to the successful treatment of breast and ovarian cancers as well as a number of other neoplasms. Chief among the causes of drug resistance is the overproduction of P-glycoprotein (P-gp), which functions to pump cytotoxic agents out of tumor cells thereby decreasing their therapeutic potential.

For nearly two decades, scientists and clinicians have explored pump antagonists to counteract efflux of chemotherapeutic agents from the cell; however, clinical responses have not been satisfactory. P-gp inhibitors are still under investigation, but we believe that successful suppression of drug resistance requires interventions that limit production of P-gp.

Because levels of glucosylceramide (GC) are elevated in adriamycin-resistant cancer cells including breast cancer cells, this study examined the influence of glycolipids such as GC on expression of the multidrug resistance gene (*MDR1*). We found that inhibition of cellular GC synthesis decreased expression of *MDR1*. Thus, exposure of adriamycin-resistant breast cancer cells (MCF-7-AdrR) to a specific inhibitor of glucosylceramide synthase (GCS), which is the enzyme catalyzing GC synthesis, or to tamoxifen (5.0 μM), a nonspecific inhibitor of GC synthesis, decreased or eliminated expression of *MDR1*. Further, exposure of cells to GCS small interfering RNA (siRNA, 100 nM) for 48 hours not only eliminated GCS expression but also reduced *MDR1* expression as measured by RT-PCR. Targeting the synthesis of GC by inhibitors or by RNA interference additionally increased the cytotoxicity of natural product chemotherapy by preserving higher intracellular drug levels.

To further examine our hypothesis that expression of *MDR1* is controlled by glycolipids, we exposed wild-type, *MDR1*-poor breast cancer cells to a cell-permeable GC analog (C8-CG, 10 μg/ml medium); after 3 days, *MDR1* mRNA increased twofold in MCF-7 cells and fourfold in MDA-MB-231 cells as compared with untreated control cells (real-time RT-PCR).

In conclusion, our study is the first to attack multidrug resistance by manipulating the levels of glycolipids through GCS. Further research will examine how reducing P-gp burden or preventing the onset of drug resistance caused by MDR1/P-gp might affect the clinical response to anthracyclines.

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P20-13: LYSOSOME-MEDIATED CELL DEATH AND AUTOPHAGY-DEPENDENT MULTIDRUG RESISTANCE IN BREAST CANCER

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Drug resistance remains the most serious limiting factor in modern oncology when a tumor previously sensitive to drug therapy no longer responds to treatment. To find genes that cause resistance to a tamoxifen, we introduced a full-length cDNA library into MCF7 cells and exposed the resulting population to cytotoxic dose of 4-hydroxytamoxifen. Selected genes were investigated: all of them caused better survival of MCF7 cells after treatment with 4-hydroxytamoxifen, thus representing true resistance-inducing genes (RIGs). Surprisingly, expression of RIGs improved viability of untreated MCF7 cells as well by stabilizing mitochondria, reducing the average number of active lysosomes, and improving stability of plasma membrane. As a result, RIG-expressing MCF7 became more viable in general stress conditions (high density of growth, lack of nutrients, etc.). When tested with different chemotherapeutic drugs, RIG-expressing cells better survived treatment with melphalan and mafosfamide but were more sensitive to 5-fluorouracil; there was no difference in sensitivity to other tested drugs.

Reduced activity of lysosomes in RIG-expressing cells suggests that RIGs interfere with autophagic cell death, which is executed through lysosome-mediated self-digestion of affected cells. In this project we proposed to investigate cell death regulated by RIGs and to define some of the regulatory mechanisms of this process. The following are the specific aims of the project: (1) to compare relative abundance and tamoxifen-induced changes of different components of autophagosomal death-related organelles in parental MCF7 cells and in their resistant derivatives, (2) to compare pathway-specific expression profiles of parental MCF7 cells and their resistant derivatives in their native state and after treatment with tamoxifen, and (3) to functionally test the role of key up- or downregulated genes in autophagy-dependent multidrug resistance. The first aim will produce critical evidence on the cell biology of RIG-affected cell death, defining which component of lysosomal activity is required for its execution. The second aim will define molecular events that take place to regulate this type of cell death while the third aim will confirm molecular findings in the context of cell biology.

In the first 6 months since the beginning of the project, we completed the development of expression profiles for parental MCF7 cells and their resistant derivatives with and without treatment with tamoxifen. Identification of the affected pathways is currently being done; results of the analysis will be reported.

We expect that identified pathways will reveal key targets for small molecule drug development that will either prevent development of resistance to tamoxifen or will reverse its course.

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P20-14: A NEW MECHANISM FOR ESTROGEN-STARVATION RESISTANCE IN BREAST CANCER

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Background and Objectives: The recent development of aromatase inhibitors as effective treatment for estrogen-receptor positive breast cancer represents a major advance in improving breast cancer therapy. However, cancer cells often acquire adaptations that result in resistance. Estrogen-starvation induced apoptosis of breast cancer cells requires BIK, an apoptotic BH3-only protein located primarily at the endoplasmic reticulum (ER). In search of novel partners that interact with BIK at the ER, we discovered that glucose-regulated protein GRP78/BiP, which is upregulated in about 70% of breast tumors, forms complexes with endogenous BIK. GRP78 overexpression decreases apoptosis induced by ER-targeted BIK in human cell cultures. For estrogen-dependent MCF-7/BUS breast cancer cells, overexpression of GRP78 inhibits estrogen-starvation-induced Bax activation, mitochondrial permeability transition, and subsequent apoptosis. Furthermore, knockdown of endogenous GRP78 by siRNA sensitizes MCF-7/BUS cells to estrogen-starvation-induced apoptosis. This project aims to test whether: (1) suppression of GRP78 will re-sensitize estrogen-receptor positive breast cancer cells that have acquired resistance to estrogen-starvation therapy; and (2) GRP78 inhibits the proapoptotic activity of BIK through interference of its cooperation with other members of the BH3-protein family.

Methods: Aim 1 is to isolate a panel of human breast cancer cells that are resistant to estrogen-starvation-induced apoptosis. MCF-7/BUS cells were cultured in estrogen-starvation medium (DMEM supplemented with 5% charcoal-dextran stripped fetal calf serum) for 5 to 10 days so that about 10% of cells survived. The surviving MCF-7/BUS cells were cultured in regular DMEM with 5% fetal calf serum for 2 to 3 days to allow expansion of remaining cells. This selection process was repeated 6 times to obtain resistant clones that could grow and divide in the estrogen-starvation medium. The resistant clones of MCF-7/BUS were maintained in this medium for at least 2 months to stabilize resistant properties before being analyzed for ER chaperone levels and ER stress markers. Our plan is to introduce siRNA targeted against GRP78 and/or other induced chaperones into the resistant cells and test whether lowering their levels will re-sensitize the resistance cells to estrogen-starvation-induced apoptosis. In Aim 2, we will determine the interactive domains between GRP78 and BIK. In Aim 3, we will examine the mechanisms whereby GRP78 inhibits estrogen-starvation-induced cell death. This will include testing: (a) whether GRP78 suppresses the cooperative mechanism between NOXA and BIK, both of which are pro-apoptotic; (b) whether GRP78 releases the pro-survival BCL-2 from BIK; and (c) whether GRP78 suppresses caspase activation by estrogen starvation.

Results: We have obtained 14 resistant clones and observed prominent elevation of ER chaperones in 9 of the clones and will proceed with the proposed experiments.

Conclusion: Our preliminary results suggest that induction of the cytoprotective ER chaperone is an adaptive mechanism to allow breast cancer cells to be resistant to estrogen-starvation. If this is validated, drugs that can suppress ER chaperones in breast tumors will sensitize the cancer cells to aromatase inhibitor therapy.

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P20-15: MICROTUBULE ASSOCIATED PROTEIN Tau AND CELLULAR SENSITIVITY TO TAXANES: MECHANISM, MARKER, OR EPIPHENOMENON?

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Microtubule associated protein (MAP) Tau, an alternatively spliced protein primarily expressed in neurons, is associated with clinical resistance to taxane drugs in breast cancers. We hypothesized that expression levels of Tau isoforms confer altered taxane response as a result of changing the properties of the drugs' target, the microtubules. Our laboratory has derived several taxane resistant breast cancer variants from MCF-7, MDA-MB-231, BT-549, and T-47D parental cells by continuous exposure to docetaxel and the MDR1 (ABCB1)/P-glycoprotein inhibitor PSC-833. RT-PCR assays confirmed that these variants are negative for MDR1 transcripts, indicating that these cells became resistant to taxanes via MDR1-independent mechanisms. We have determined the expression levels of each Tau isoform by both real-time PCR and western blotting using isoform specific primers and antibodies. Our results show that the T-47D and MDA-MB-231 taxane-resistant variants express significantly higher levels of all six major MAP-Tau isoforms compared to the parental cells. In addition, both the parental cells and taxane-resistant variants express Tau exon 6, a finding not reported for normal brain tissue. To evaluate whether down-regulation of Tau increases the sensitivity of MCF-7 cells to taxanes, we knocked down the expression of Tau using anti-Tau shRNA delivered by lentiviral infection. Decreased Tau expression did not increase the sensitivity of MCF-7 cells to taxanes as determined by both SRB cell proliferation and clonogenic assays. This result was substantiated in OVCAR-3 ovarian cancer cells, which also express Tau. Tau-silenced cells exposed to various concentrations of taxanes did not show any alterations in tubulin polymerization, and there was no difference in [³H]-docetaxel binding in Tau-silenced relative to control cells. In conclusion, we have demonstrated that some taxane-resistant breast cancer cells show overexpression of all six major Tau isoforms; however, Tau silencing is not sufficient to increase taxane sensitivity, alter taxane binding to microtubules, or alter tubulin polymerization. We are currently evaluating whether Tau overexpression is a downstream event of estrogen receptor signaling and whether such overexpression is sufficient to increase taxane resistance.

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P20-16: THE NFκB INHIBITOR PARTHENOLIDE RESTORES SENSITIVITY TO 4-HYDROXY TAMOXIFEN THROUGH APOPTOTIC PATHWAYS INVOLVING TNFR1 AND Bcl-2 IN ANTIESTROGEN-RESISTANT MCF-7 BREAST CANCER CELLS

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Breast tumors are initially dependent on estrogen for growth. Both steroidal (e.g., ICI 182,780; Faslodex) and nonsteroidal (e.g., Tamoxifen) antiestrogens can inhibit estrogenic effects in breast tissues. However, acquired drug resistance is a major limitation to their effectiveness in breast cancer. Precise mechanisms that contribute to acquired

resistance remain undefined. NF κ B, a transcription factor, may play an important role in breast cancer and specifically in antiestrogen resistance. Thus, it is important to establish the functional role of increased NF κ B activity in the antiestrogen resistant phenotype, and whether interfering with NF κ B activity might provide a means to improve existing antiestrogen therapy. We have previously reported that p65 NF κ B expression and activity is elevated in MCF7/LCC9 breast cancer cells that have acquired resistance to 4-hydroxy tamoxifen (4HT) and ICI 182,780 (ICI; FAS). We hypothesize that upregulation of NF κ B's transcriptional activity contributes to an antiestrogen resistant phenotype, whereas a reduced activation would provide a means to overcome resistance and re-establish sensitivity to antiestrogens.

We have previously reported that NF κ B inhibition by the small molecule inhibitor parthenolide restores sensitivity to the steroidal antiestrogen FAS. Most recently, we have found that parthenolide as well as a dominant negative NF κ B inhibitor (IkBSR) are both very effective in downregulating NF κ B's activity and restoring sensitivity to the nonsteroidal antiestrogen Tamoxifen. Specifically, while MCF7/LCC9 breast cancer cells are unresponsive to 4HT alone they are sensitive to parthenolide, and a com-

bination of 4HT and parthenolide results in significant sensitization of MCF7/LCC9 cells to 4HT. Restoration of sensitivity to 4HT is accompanied by increased apoptosis and mitochondrial membrane permeability, and decreased expression of PARP in the antiestrogen resistant MCF7/LCC9 cells. Because parthenolide can activate c-Jun N terminal kinase (JNK) independent of NF κ B inhibition, we examined if restoration of antiestrogen sensitivity in MCF7/LCC9 cells is a result of parthenolide-induced activation of JNK. We found that protein levels of JNK and phospho-JNK do not change in MCF7/LCC9 cells irrespective of the treatment, suggesting that the synergistic interaction between parthenolide and antiestrogens does not involve activation of the JNK pathway. Interestingly, parthenolide and 4HT do synergize to decrease expression of the anti-apoptotic protein Bcl-2 and TNFR1, thereby restoring 4HT-induced apoptosis in MCF7/LCC9 cells. Taken together our findings suggest that downregulation of NF κ B reverses the antiestrogen resistant phenotype and provide support for designing clinical trials to combine parthenolide and antiestrogens in patients.

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BIOMARKERS I

Poster Session P21

P21-1: MAMMOGRAPHIC DENSITY DOES NOT PREDICT RESPONSE TO TAMOXIFEN

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Background: Mammographic density has been validated as an independent marker of short-term breast cancer risk and a surrogate marker of response to a variety of prevention agents. Although a majority of breast cancers are epithelial in origin, there is evidence that stromal content of the breast is the primary predictor of mammographic density. This suggests that stroma may play an important role in breast carcinogenesis. However, currently we lack an understanding of how mammographic density is affected by the individual contribution of epithelial and stromal components.

Methods: Random periareolar fine needle aspiration (RPFNA) is a research technique that has been prospectively validated to assess (1) short-term breast cancer risk and (2) response to chemoprevention in high-risk women. We used a combination of RPFNA and mammographic density to test for response to tamoxifen prevention in 20 high-risk premenopausal women with mammary atypia and 20 controls who declined tamoxifen.

Results: We observed that 11/20 women had disappearance of atypia on RPFNA after 12 mos tamoxifen and 9/20 women had persistent atypia. Interestingly, a portion of women with persistent atypia had (1) a marked decrease in mammographic density, (2) a corresponding decrease in RPFNA stromal counts but (3) no decrease in epithelial cell counts.

Conclusions: Our observations suggest that the stroma and epithelium may not respond in synchrony to prevention agents, and therefore, mammographic density may not be a reliable measure of epithelial response. We hypothesize that in the normal breast, the stroma and epithelium act in synchrony to regulate mammary gland homeostasis and breast density. During early mammary carcinogenesis (hyperplasia and low-risk atypia), there is increased stromal/epithelial proliferation and mammographic density. Under these circumstances, we predict that response to a prevention agent would be synchronous, and therefore, mammographic density would be a reliable measure of response. However, in high-risk atypia, we hypothesize that the epithelium and/or stroma may acquire sufficient epigenetic/genetic damage to proliferate independently. Under these circumstances, we predict that response to a prevention agent could be synchronous, asynchronous, or nonresponsive; therefore, mammographic density may not be a reliable measure of response.

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P21-2: E-CADHERIN LEVELS PREDICT OUTCOME IN INFLAMMATORY BREAST CANCER

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George Washington University

Background: Inflammatory Breast Cancer (IBC) is the most aggressive and lethal form of breast cancer. One of the most common biomarkers implicated in IBC is e-cadherin overexpression in the tumor cells. In our earlier study of tumor markers in IBC (Portera et al., *SABCS* 2006) mean e-cadherin expression was higher in IBC patients than controls but there was wide individual variation. In this report we describe the relationship of clinical parameters to e-cadherin levels.

Materials and Methods: The e-cadherin in all 48 IBC Registry patients with pathologic confirmation (involvement of the dermal lymphatics) that were part of our earlier study were divided into two groups as high (>2.0 , $n=24$) or low ($0-2$, $n=24$) levels. Their responsiveness to chemotherapy was assessed based on the presence of pathological evidence of residual tumor at mastectomy and both patient's and the oncologist's assessment of the clinical response to chemotherapy. The responses were broadly divided into excellent/good, partial, and none/poor responses. Comparative analysis also included Her2neu, ER, PR status, progression-free survival (PFS) and overall-survival (OS), stage at diagnosis and presence of metastasis.

Results: All patients in the low e-cadherin expression category had either an excellent/good response to chemotherapy or a partial response. In the high level category X (y%) showed none/partial response to chemotherapy. Patients with higher levels were more likely to present with metastatic disease (62% versus 31%) and be Her2/neu positive (62% versus 37%). No difference was seen regarding ER/PR receptors. PFS and OS being determined.

Discussion: The results of this study indicate that e-cadherin overexpression is associated with increased aggressiveness of pathologically confirmed IBC. It is speculated that increased adhesiveness of the cells facilitates the establishment of microemboli.

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P21-3: A PROSPECTIVE STUDY OF MULLERIAN INHIBITING SUBSTANCE AND BREAST CANCER RISK

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Background and Objectives: Mullerian inhibiting substance (MIS) is a member of the transforming growth factor β family of growth and differentiation factors known primarily for its role in regulating in utero sexual differentiation of males. In the female breast, MIS inhibits elongation and branching of mammary ducts and growth of breast tumors. Thus, MIS is a novel biomarker that could potentially decrease breast cancer risk. MIS is produced by the ovaries and signals via the MIS receptor type II, which in humans is expressed by normal breast epithelial cells as well as ductal carcinomas [1]. MIS plays a key role in regulating normal development of the ductal architecture of the breast. In particular, MIS stimulates apoptosis of breast ductal epithelium [2] and in vivo inhibits proliferation of mammary ducts [3]. MIS also has direct effects on breast tumor growth. In vitro, it inhibits growth of breast cancer cells via inhibition of cell cycle progression and induction of apoptosis [1]. Thus, MIS could decrease breast cancer risk by effects on the ductal architecture and local environment of the breast or through direct effects on tumor growth. The primary objective of this study is to prospectively determine if MIS levels in serum are related to breast cancer risk.

Methods: A case-control study will be performed nested in the Columbia, Missouri Serum Bank. Each of 125 cases with prediagnostic serum collected before menopause will be selected matched to 2 controls on age, date, menstrual cycle day, and time of day of blood collection. MIS will be measured using an enzyme linked immunosorbent assay. Because MIS correlates with testosterone in serum and inhibits ovarian aromatase activity [4], we will measure serum testosterone and estradiol by radioimmunoassay to allow for adjustment in analysis. Data analysis will be performed using conditional logistic regression. We will have 80% power to detect at $p_{2-sided} < .05$ an odds ratio of 1.86 when comparing individuals above and below the median MIS value.

Results: Results will be presented at the 2008 Era of Hope Meeting.

Conclusions: The proposed study could identify a novel serum biomarker associated with breast cancer risk and could open new avenues for investigation into breast cancer etiology and prevention.

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P21-4: POTENTIAL PROGNOSTIC AND THERAPEUTIC VALUE OF Rad6 IN BREAST CANCER

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The Rad6 gene is a fundamental component of postreplication DNA repair, and its ubiquitin conjugating activity is essential for its function. We have previously shown that Rad6B is minimally expressed in normal human breast tissues, and detectable increases in Rad6B expression occur in breast hyperplasia with significant overexpression in DCIS and invasive carcinomas. We have also shown that the ability of human breast cells to tolerate DNA damage induced by chemotherapeutic drugs correlates with Rad6B expression levels and postreplication DNA repair capacity. To determine if Rad6B protein expression levels and distribution can be used singly or in combination with p53, Mdr-1, or PCNA as a predictor of response to neoadjuvant chemotherapy, we performed a retrospective analysis of posttreatment surgical excisions from 24 patients with locally advanced breast cancer. Rad6B presence in the nucleus correlated significantly with complete clinical response to neoadjuvant treatment whereas no associations between p53, PCNA, or Mdr-1 and clinical response to treatment were found. Conversely, Rad6B overexpression in the cytoplasm was significantly associated with clinical "no response" to treatment. These data suggest that

elevated levels of Rad6B in the cytoplasm may contribute adversely to breast cancer progression and/or diminished response to chemotherapy by inducing ubiquitin modifications of substrate proteins in the cytoplasm. cDNA array analysis performed to identify genes potentially contributing to the oncogenic effects of Rad6B overexpression revealed augmented expression of several key members of the Wnt signaling pathway. A positive relationship between endogenous Rad6B protein levels, β -catenin ubiquitination status, and β -catenin-mediated transcriptional activity was found in breast cancer cells that show autocrine activation of Wnt signaling. Rad6B depletion with Rad6BshRNA resulted in suppression of β -catenin polyubiquitination, β -catenin-mediated transcriptional activity along with a visible decrease in intracellular β -catenin levels without effect on cell membrane-associated native β -catenin. In vitro ubiquitination assays using recombinant Rad6B confirmed that Rad6B catalyzes polyubiquitination of β -catenin in the absence of a ubiquitin ligase, and Rad6B ubiquitinated β -catenin is insensitive to degradation by 26S proteasome. The human Rad6B gene is a target of transcriptional activation by β -catenin/TCF/p300 through a TCF binding site in the Rad6B promoter. Consistent with low Rad6B expression in normal breast cells, the Rad6B promoter activity is repressed in MCF10A cells, and depression/activation of the Rad6B promoter requires coexpression of β -catenin and p300. These data suggest the operation of a positive feedback loop between Rad6B expression and β -catenin stability/transcriptional activity. More importantly, these data provide a mechanistic basis for imbalances in Rad6B levels and activity and its contribution to acquisition of drug resistance.

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P21-5: IDENTIFICATION OF NEW SERUM BIOMARKERS FOR EARLY BREAST CANCER DIAGNOSIS AND PROGNOSIS USING LIPID MICROARRAYS

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Breast cancer is the most common form of cancer among women. Compared with other serum polypeptides, autoantibodies have many appealing features as biomarkers including sensitivity, stability, and easy detection. Anti-lipid autoantibodies are routinely used in the diagnosis of autoimmune disease, but their potential for cancer diagnosis has not been explored. Metabolism of lipids immediately follows cellular stimulation, resulting in various lipid metabolites. Dysregulation of cellular signaling in cancer cells would be expected to lead to irregular metabolism of many lipids, which could be sensed by immune system and cause the production of novel autoantibodies. Indeed, recent reports describe anti-lipid antibody production in cancer patients.

Our hypothesis is that a broad and irregular change in lipid profiles in breast cancer cells results in the production of anti-lipid antibodies that could be used as biomarkers for early diagnosis. We proposed to generate arrayed lipids on the membranes (lipid microarrays), and use them to examine global anti-lipid profiles at different stages of carcinogenesis in a transgenic breast cancer model. We have successfully improved the technology for lipid microarrays in our laboratory. Using fluorescently labeled secondary antibodies and an internal control labeled with a different fluorophore, we have greatly improved the sensitivity and reproducibility compared to the first generation of lipid microarrays. We are currently testing the anti-lipid responses using sera collected from the normal and Her2 transgenic breast cancer mouse models.

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P21-6: PERIPHERAL BLOOD METHYLATION: A POSSIBLE BIOMARKER FOR PREDICTING BREAST CANCER RISK

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Peter MacCallum Cancer Centre

Background and Objectives: We are examining the concept that individuals vary in their propensity to methylate promoter CpG islands. Individuals more prone to methylate their CpG islands may be more susceptible to breast cancer. Our objective is to test peripheral blood for promoter methylation of genes known to be methylated in breast cancer using sensitive quantitative assays to determine an index of methylation in somatic tissues and to relate this to breast cancer predisposition. We also aim to determine whether individual differences in methylation of normal tissues are related to polymorphisms in genes involved in the control of methyl metabolism.

Brief Description of Methodologies: Two methodologies were developed during the course of this study to accurately assess low levels of methylation. The first was methylation-sensitive, high-resolution melting, which amplified sequences regardless of their methylation status and then used high-resolution melting to differentiate between fully, partially, and unmethylated sequences. The second methodology was a new quantitative variant of methylation-specific PCR (MSP) that was able to identify false-positive results. A third methodology based on high-resolution melting was developed to rapidly genotype DNA samples for single nucleotide polymorphisms.

Results to Date: Although methylation-sensitive, high-resolution melting assay could be sensitive to as low as 0.1% methylation, heterogeneous methylation reduced the effective sensitivity of the assay. The quantitative variant of MSP proved to be routinely sensitive to 0.1% methylation and was more sensitive in detecting methylation in patient samples.

Conclusions: Our study differs from other studies in that we take a clearly uninvolved tissue, peripheral blood leukocytes, to determine the inherent epigenetic instability of an individual. Our results show that using sensitive assays, low-level methylation is detectable for many genes. This can be a possible biomarker for breast cancer risk. The sensitive methylation assays developed would also be of use for monitoring the response of breast cancer to therapy.

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P21-7: IDENTIFICATION OF MOLECULAR MARKERS FOR BREAST METASTASIS

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The clinical outcome is generally positive for patients with node-negative breast carcinoma. However, in about 30% of the lymph node negative patients, the disease spreads, and they are at risk of death. The main objective of this project is to develop panels of genetic markers that could diagnose 30% of mammary carcinomas (Group-I) that are prone to developing metastases and the remaining 70% breast cancers (Group-II) that are not likely to metastasize. Previously we subjected DNA samples isolated from cells in the breast tissue (normal cells/primary tumor) with that of the cancer cells in the lymph nodes of 20 patients to representational difference analyses (RDA) and identified novel 15 metastasis-associated DNA sequences (MADSs). Our studies using PCR and FISH methods showed that these MADSs are lost (heterozygous/homozygous) in the metastatic lymph node samples of a considerable number of samples we tested. We therefore hypothesized that the MADSs themselves could be part of the genes involved in metastasis and/or the chromosomal regions surrounding these MADSs may harbor genes that are involved in the suppression of metastasis. In support of our second part of hypothesis, we found several genes in the regions surrounding these MADSs that are known to be associated with different pathways of cancers and metastasis. Presently we propose to screen such genes on the archival primary breast cancer tissue samples (whose clinical outcome is known) at RNA and protein levels to identify panels of robust candidate molecular markers which can reliably differentiate group-I breast cancers from those of group-II. For the proposed aim, we selected a set of 20 genes located in the vicinity of MADSs, designed primers and standardized conditions for real-time RT-PCR and immunohistochemistry studies for those 20 genes/proteins and also obtained 80 archival primary tumor samples of patients with breast cancer [(Group-I with positive lymph nodes: 40) + (Group-II with negative lymph nodes: 40)]. This year we also have submitted this proposal to Susan G. Komen Breast Cancer Foundation for funding. To test our working hypothesis, recently we selected 5 genes randomly from those 20 genes namely BUB3, PTEN, ANXA7, FOXO3A1, and IGF1R and performed semiquantitative RT-PCR on 2 non-metastatic breast carcinoma cell lines (MCF-7 and BT-474) and a highly metastatic cell line (MDA-MB-435). While IGF1R and PTEN showed under expression (about 2.7; 2.9 folds) in highly metastatic cell line (MDA-MB-435) as compared to non-metastatic cell lines, BT-474 and MCF-7, other 3 genes did not show any significant differences in these 3 cell lines. Western blot experiments to validate these findings are in progress. These preliminary results reiterated the involvement of PTEN in breast metastasis and also indicated IGF1R as a possible marker for breast cancer/metastasis. Currently, similar studies using archival breast tumor samples are under way. Breast carcinoma cell lines that show low levels of expression of gene(s) can be used as in vitro/in vivo model(s) to validate the metastatic potential of the gene(s) that we may find from this study. We believe that our proposed RNA and protein expression of 20 genes using the archival primary tumor samples in this project should provide us with a panel of reliable gene markers specific to Group-I and Group-II breast tumors. Such molecular markers could be further validated in a prospective clinical study at Temple University Hospital.

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P21-8: TARGETING THERAPY-RESISTANT TUMOR VESSELS

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Burnham Institute

Preventing tumor angiogenesis has become a promising new form of cancer therapy, including that of breast cancer. However, researchers have found that such therapy preferentially prunes the immature vessels in solid tumors, leaving behind a more "normal" vasculature, which can maintain and even improve tumor blood flow and oxygenation. The purpose of this project is to develop molecular markers for these therapy-resistant vessels. We use in vivo phage display of peptides to search for

molecular markers in blood vessels (Ruoslahti, *Nature Reviews Cancer*, 2, 81-90, 2002); our method primarily targets the endothelium of the vessels. The anti-angiogenic treatment we use is administering tumor mice with anti-nucleolin antibody. We have shown that cell surface-expressed nucleolin is a marker of angiogenesis (Christian et al., *J. Cell Biol.* 163, 871-878, 2003) and that the antibody can reduce tumor vessel density by as much as 60%, while having no effect on tumor growth. Moreover, the treatment produces a morphologically normalized tumor vasculature as detected by increased perivascular cell and basement membrane coverage and decreased vascular lumen and tortuosity. This structural normalization of tumor vessels translates into functional normalization of the vasculature, as anti-nucleolin treatment decreases tumor hypoxia, likely by improving the quality of tumor vessels. These results are in agreement with recent findings on vessel "normalization" in tumors treated with other anti-angiogenic compounds. As there is no effect on tumor growth, anti-nucleolin treatment appears to eliminate only immature vessels, leaving behind the more mature and functional vessels. We are exploiting the selective nature of the vascular regression brought about by anti-nucleolin treatment in a search for novel markers of the vessels that remain and nurture the tumor after anti-angiogenic therapies. We screen phage-displayed peptide libraries for peptides that home to the treated tumors. Our goal is to identify peptides that recognize the currently treatment-resistant tumor vessels and to use these peptides to specifically target the resistant vessels for destruction. We are employing two strategies in search of homing peptides and markers for therapy-resistant vessels: (1) Testing of a large panel of tumor-homing peptides we have developed that specifically recognizes changes in tumor vessels and (2) phage library screening of tumors that has been treated with anti-angiogenic therapy. We have identified promising candidates among our existing panel of tumor-homing peptides. We are now testing the homing of these peptides to anti-nucleolin-treated tumors and analyzing their localization in tumor vessels relative to vascular maturity markers such as alpha smooth muscle actin, the pericyte marker NG2, and laminin (a basement membrane component). We have also developed the procedures for new phage library screens that use treated tumors. These results will be described in detail at the meeting. Availability of markers for therapy-resistant tumor vessels is expected to be useful in developing strategies for a more extensive destruction of breast cancer vasculature, than is currently possible. Improvements in breast cancer therapy may ensue.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0482.

P21-9: DEVELOPMENT OF A SIMPLE CLINICAL ASSAY FOR MEASUREMENT OF BREAST CANCER CELL MICROVESICLES IN PLASMA

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Cellular microvesicles (MV) are secreted membrane particles of about 100-200 nm in size. MV are derived from platelets, endothelial cells, white blood cells, and solid tumor cells. Tumor MV have protean pathophysiological activities including pro-angiogenic, pro-metastatic, pro-inflammatory, and pro-coagulant properties, and the immunologic induction of tumor tolerance. It is likely that elevated levels of tumor cell MV in the blood will be found to correlate with increased tumor malignancy. However, a clinically useful assay for tumor MV has been difficult to develop. Previous work has involved the use of flow cytometry to study these viral-sized particles. Flow cytometry is only able to characterize the protein expression on MV. Flow cytometry is unable to quantitate MV in any way due to physical limitations of the laser wavelength of currently available machines. We have developed a simple quantitative clinical assay for breast cancer cell MV that does not involve flow cytometry. Our assay also avoids problems related to MV preparation by high speed differential centrifugation (artificial fusion of MV during sedimentation), or freeze-thawing of clinical samples (artificial lysis of MV). Citrated blood is centrifuged to obtain platelet-free plasma (PFP). Due to their small size, the tumor MV remain in the PFP. An aliquot of the PFP is syringe-filtered using 100 nm cut-off filters. Both filtered and unfiltered PFP samples are incubated for 1 hour at room temperature with magnetic beads coated with antibodies for a tumor antigen of interest. The beads are magnetically captured and washed. The beads (with the adherent tumor MV) are subjected to detergent lysis overnight at 4°C in the presence of protease inhibitors. The lysis fraction is then quantitatively assayed for a tumor antigen of interest using ELISA. The arithmetic difference in ELISA values between the filtered and unfiltered sample represents the contribution of the tumor MV. We currently are characterizing this assay using anti-tissue factor (TF) as the capture antibody and performing a TF ELISA for quantitation. We have shown expression of TF on the surface of breast cancer MV. Our assay is able to quantify purified tumor MV from MDA-MB-231 cells spiked into normal human plasma. We anticipate using our assay as a platform for various specific MV assays. For instance, we will capture MUC1-expressing breast cancer MV in plasma, and subsequently assay for TF or MMP-9 by ELISA. We have demonstrated expression of both MUC1 and MMP-9 on human breast cancer MV. We have demonstrated both DNA and RNA in MDA-MB-231 MV. Thus, our bead capture assay also allows the development of novel assays such as mutagenic analysis of genes of interest in the tumor MV during and after treatment. We are currently prospectively obtaining tumor MV lysates from patients enrolled in a UAB neoadjuvant Phase II study of letrozole versus letrozole in combination with bevacizumab

in post-menopausal women with newly diagnosed operable breast cancer. We hypothesize that disappearance of plasma tumor MV will correlate with pathologic response at surgery. We suspect that, in the future, tumor MV will be shown to be not just a valuable biomarker but a major contributor to metastasis. Any attempts to clinically intervene to decrease tumor MV will require a quantitative assay such as we describe here.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0420.

P21-10: PROTEOMIC PREDICTION OF BREAST CANCER RISK

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The pathological processes that underlie disease development are fundamentally protein-driven. Proteomics is being used increasingly to investigate pathological processes. To date, such investigations have mostly been conducted as cross-sectional studies. Our objective is to extend the use of proteomic technology to the prediction of risk of subsequent invasive breast cancer. Our underlying hypothesis is that proteomic analysis of serum will identify proteins differentially expressed in women who develop invasive breast cancer versus those who do not and that these differences will be identifiable several years prior to the clinical presentation of breast cancer.

Our work is being conducted in two stages, a training stage followed by a validation stage, each entailing a case-control study nested within a population-based cohort of women from whom serum samples were collected between 1985 and 1992. The training stage involves 40 case-control pairs and the validation stage will involve 20 case-control pairs. Each stage will involve analysis of serum samples from cases, matched controls, and a serum pool created by combining sera from all subjects selected for both stages of the study. The laboratory staff are blinded to case/control status.

For proteomic analysis, serum (20 µl) from each of the 3 samples within one stratum are loaded separately onto an immunoaffinity protein depletion column to remove the 12 major serum proteins, including albumin and immunoglobulin. The depleted serum is digested with trypsin. The 3 peptide preparations are each labeled with a different iTRAQ reagent (114, 115, or 116), then mixed and subjected to strong cation exchange (SCX) chromatography to separate the labeled peptides into 6 fractions according to their charges. Peptides in each SCX fraction are further separated into 192 fractions using capillary C18 reversed phase liquid chromatography. These fractions are collected in-line with a robotic collector onto 192-well MALDI plate, concurrently mixing each with matrix a-CHCA. Each of the fractions is then subjected to MALDI-TOF-MS analysis to generate a list of parent ions. Up to 25 parent ions from each fraction are further subjected to MALDI-TOF-MS/MS analysis. Each MS/MS spectrum contains fragmentation ion information and iTRAQ reporter ion information. The fragmentation ion information is used for protein identification. The areas of the peaks of the reporter ions 114, 115, and 116 are used for relative quantification of the three samples within a stratum. Our preliminary results from analysis of the first 7 strata indicate detection of about 2000 proteins with high confidence (Mascot score > 36), including several proteins of low abundance, such as interleukins. Updated results will be presented at the meeting.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0298.

P21-11: SERUM BIOMARKER PROFILES AND RESPONSE TO NEOADJUVANT CHEMOTHERAPY FOR LOCALLY ADVANCED BREAST CANCER

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University of Pittsburgh

Neoadjuvant chemotherapy has become the standard of care in locally advanced breast cancers. This type of treatment offers certain distinct advantages including the ability to evaluate in vivo the chemosensitivity of the tumor. Gene expression profiles and serum xMAP™ technology offers the ability to measure multiple serum biomarkers. Biomarker levels are increasingly being investigated for their ability to predict therapy response and aid in the development of individualized treatment regimens. The Luminex quickly form a small amount of starting material. Multianalyte profiles may offer greater predictive power for neoadjuvant treatment response than the individual biomarkers currently in use. Serum samples were collected from 44 patients enrolled in a Phase I-II, open-label study of liposomal doxorubicin and paclitaxel in combination with whole-breast hyperthermia for the neoadjuvant treatment of locally advanced breast cancer (stage IIB or III). Samples were collected prior to each of four rounds of treatment and prior to definitive surgery. Samples were assayed by Luminex for 55 serum biomarkers including: cancer antigens, growth/angiogenic factors, apoptosis-related molecules, metastasis-related molecules, adhesion molecules, adipokines, cytokines, chemokines, hormones, and other proteins. Biomarker levels were compared retrospectively with clinical and pathologic treatment response.

Univariate analysis of the data identified several groups of biomarkers that differed significantly among treatment outcome groups early in the course of neoadjuvant chemotherapy. Multivariate statistical analysis revealed several multi-biomarker panels that could differentiate between treatment response groups with high sensitivity and specificity. We demonstrate here that serum biomarker profiles may offer predictive power concerning treatment response and outcome in the neoadjuvant setting. The continued development of these findings will be of considerable clinical utility in the design of treatment regimens for individual breast cancer patients.

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P21-12: MICROARRAY ANALYSIS OF CpG METHYLATION AND ALTERED GENE EXPRESSION IN THE MCF10A MODEL OF BREAST CANCER PROGRESSION

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DNA methylation results in transcriptional repression of tumor suppressor genes in a variety of cancers. Our objective was to develop a profile of epigenetic changes occurring during the early stages of breast cancer progression, as modeled using the MCF10 series of human breast cells. Our hypothesis is that a simpler, interpretable pattern of changes can be derived by studying the changes that occur in these breast cells cultured from a single individual as they undergo changes from normal appearing ductal forms, through hyperplasia to the appearance of carcinoma-in-situ and locally invasive cancer. We have previously reported on the methylation status and expression profiles of seven marker genes known to be altered in cancer using these cell lines. Analysis of gene expression using Affymetrix U133 Plus2 arrays detected changes in expression of these genes that were consistent with data obtained by RT-qPCR and identified a number of other genes whose expression in breast tumors has been reported [1]. However, while analysis of methylation status of CpG islands using CpG island arrays (Human 12K microarray, Microarray Centre, Ontario Cancer Institute) indicated differences in the extent of methylation between the cell lines and a general trend toward increased CpG island methylation, only a few known genes were detected that had significant increases or decreases in methylation that correlated with changes in gene expression.

We have now repeated array analysis of DNA methylation in two primary breast lines and the tumorigenic lines derived from MCF10A using the Affymetrix Human 1.0R promoter array. Since this array allows examination of CpG methylation of over 1,300 cancer associated genes and contains ~59% of UCSC annotated CpG islands, the yield of useful data is greatly enhanced relative to that from the 12K array. Initial results indicate significant gain of methylation in both CpG islands and other CpG rich regions in all of the MCF10AT (tumor forming) lines with MCF10CA1h displaying the greatest increase in methylation (>500 regions). Areas of CpG methylation loss were also common in the three lines. A summary of the most important findings arising from our array analysis will be presented. An example is imprinted gene, NOEY2, a gene expressed in normal breast tissue. The unmethylated, expressed allele is known to be methylated and silenced in several breast cancer cell lines [2,3]. We found the promoter region to be fully methylated and silenced in MCF10 cells and all of the MCF10 derived lines studied using both standard and array analysis. Our array studies reliably detected both the reduction of NOEY2 expression in MCF10A cells (relative to expression in normal breast cells) and an increased level of CpG methylation in the promoter region. This suggests (1) that NOEY2 silencing may be an early marker for breast cancer development, occurring before the cells are able to form even slow growing tumors and (2) that use of array technology should allow identification of additional early markers of breast cancer development.

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P21-13: PREVENTION OF 40%–50% OF BREAST CANCERS BY DETECTING TRUE PRECANCEROUS LESIONS AMONG BENIGN PATIENTS BASED ON THE PRESENCE OF CANCER PROMOTING MOLECULAR MARKERS

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Howard University

Surveys show that about 1–1.2 million women are diagnosed with some form of benign breast lesions per year in the USA, and 8%–9% (80,000 to 90,000) of them will subsequently develop breast cancer (NEJM 2005). If we can exactly identify women with which subtype of benign lesions will subsequently develop cancer and treat them, then we can effectively prevent a large percentage (40%–50%) of breast cancers and

deaths from it. However, it has not been possible to precisely identify a “true precancerous lesion” among benign tissues and predict which patients will subsequently develop cancer based on morphology. Molecular markers that can precisely detect a “true precancerous lesion” among benign lesions will be highly valuable to identify very high-risk benign patients for prophylactic therapies and prevention.

We took a rational approach to identify the predictive molecular markers. We hypothesized that “benign tissues from patients who developed cancer are the true precancerous tissues” and those tissues have elevated expression of a number of cancer-promoting molecules. To test the above, we compared the global gene expression profiles of ADH tissues from patients who developed cancer (ADHC) with ADH tissues from patients who had no history of cancer and identified over 300 cancer-promoting molecules in ADHC. We predicted that these molecules could be applied to distinguish the true precancerous lesions among the benign tissues once validated. We selected three highly elevated, known cancer-promoting molecular markers, MMP-1, CEACAM6, and HYAL1, among the 300 for validation. We studied the above markers in 164 archival benign tissues of various histological types by immunohistochemistry. We found that the above three markers were expressed in over 90% of tissues from patients who subsequently developed cancer in comparison to only 10%–15% of benign tissues from patients who did not develop cancer in 6–7 years irrespective of the histology of the benign lesions.

The sensitivity, specificity, PPV, and NPV for all three markers were between 0.8 and 0.9, and p values were near zero. The receiver operating characteristic curves showed areas under the curves between 0.8–0.9 for individual markers establishing that the above molecules individually are excellent markers for predicting breast cancer development in patients with benign breast lesions. We next tested if there is an advantage in making predictions based on a combination of above markers and found that the sensitivity, PPV, and NPV increased to 0.95–0.97 while maintaining the specificity. Thus, our single center study established that if a patient has the above markers in her benign tissue, she is over 95% likely to develop breast cancer subsequently. We are currently planning to conduct multicenter validation of the above three markers in a large number of samples from multi-ethnic patients across the world. We believe that once validated, the above markers could be applied to screen histologically diverse types of benign tissues and identify very high-risk benign patients for treatment with prophylactic drugs, which could drastically cut down breast cancer incidence and deaths from it.

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P21-14: MICROTUBULE-ASSOCIATED PROTEIN (MAP)-Tau IS A PROGNOSTIC BIOMARKER ASSOCIATED WITH BETTER OUTCOME IN BREAST CANCER

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Background: Clinical treatment involving adjuvant or neoadjuvant treatment currently relies on a variety of factors such as tumor size, lymph node status, degree of metastasis, and biomarker expression (ER, PR, and HER2). However, additional markers that can identify subsets of patients requiring more aggressive or pathway-targeted adjuvant treatments are needed. Microtubule-associated proteins, such as tau, have recently begun to gain attention as both predictive and prognostic markers. These proteins promote the assembly of tubulin monomers into microtubules functioning to stabilize microtubules and thus working against cancer by inducing mitotic arrest. Tau expression has been found to decrease microtubule vulnerability to taxanes, and its expression makes cells resistant to taxane treatment. In addition, low tau expression has been shown to be predictive for response to the taxane, paclitaxel, in breast cancer. However, the prognostic value of tau has not been established.

Materials and Methods: Tau expression was measured in a large retrospective breast cancer cohort (n=480) with 20-year follow-up using tissue microarray technology and automated quantitative analysis (AQUA). The AQUA system used cytokeratin to define pixels as breast cancer within the array spot and measured the intensity of tau expression using Cy5 conjugated antibodies. AQUA scores were correlated with clinical and pathologic variables.

Results: Tau showed a normal distribution of expression with high correlation (R=0.76) between redundant cores. Kaplan-Meier survival analysis with a validated optimal cut-point showed a 5-year survival rate of 82% for high expressors versus only a 60% survival rate for low expressors (log rank, P<0.0001). High tau expression correlated strongly with negative lymph node status (P=0.0007). Univariate analysis indicated a protective relationship between tau expression and outcome (OR = 0.625, 95% confidence interval [CI] = 0.52–0.75; P<0.0001).

Conclusion: Similar to microtubule-associated proteins such as tau, taxanes also bind and stabilize microtubules leading to mitotic arrest in cancer cells. Thus, taxanes may compete for binding sites with tau, and this may explain why increased tau expression results in resistance to taxane treatment (lack of functional binding sites available for paclitaxel) and why low tau expression is predictive for paclitaxel response (abundance of functional binding sites available for paclitaxel). This study found that increased tau expression is associated with better outcome. This may be reflective of increased mitotic arrest and inhibition of cellular proliferation within cancer cells that can occur when high levels of tau are present. The biological basis of high tau expression and breast cancer pathogenesis requires further investigation. These findings suggest that tau may be a useful prognostic marker in addition to its predictive value in taxane response.

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P21-15: IDENTIFY AND DEVELOP AN IN VITRO CELL MODEL WITH A HIGH LEVEL EXPRESSION OF CRIP1, A NOVEL BIOMARKER FOR BREAST CANCER

Jihua Hao and James Basilion
Case Western Reserve University

Cysteine-rich intestinal protein one (CRIP1) belongs to the LIM/double zinc finger protein family, which includes cysteine- and glycine-rich protein-1, rhombotin-1, rhombotin-2, and rhombotin-3. Human CRIP1, primarily a cytosolic protein, was cloned in 1997 using RT-PCR of human small intestine RNA and oligonucleotides whose sequence was derived from the human heart homolog of this protein, CRHP. Recently CRIP1 has been identified as a very exciting biomarker for human breast cancers. In experiments comparing CRIP1 expression in human breast cancer to matched normal breast tissue, the mRNA for this target was overexpressed 8–10-fold in approximately 90% of both invasive and ductal carcinoma in situ. Further, in situ hybridization studies demonstrated close association of the expression with the ductal carcinoma cells. Therefore, CRIP1 represents a novel pharmacological target for early detection, prevention, diagnosis, therapies, and treatment to breast cancer.

To utilize CRIP1 as a biomarker for early detection and treatment to breast cancer, an in vitro model with a high level of CRIP1 expression in breast cancer cell lines will be identified and developed. Our preliminary results have shown that CRIP1 expression in MCF-7 cells is low based on western blot analysis of protein from MCF-7 breast cancer cells. Therefore, we will screen several different breast cancer cell lines to identify a cancer cell line expressing a high level of CRIP1. Immunohistochemistry will be utilized to explore the correlation of CRIP1 protein expression to CRIP1 mRNA levels in tissue samples. If a cell line cannot be identified that expresses high levels of CRIP1, we will create one by establishing stably transfected cell lines that overexpress CRIP1 and isolate cells that most closely overexpress CRIP1 to levels representative of those measured for CRIP1 in tissues.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0700.

P21-16: IDENTIFICATION OF PEPTIDE LIGANDS TO CRIP1, A NOVEL BIOMARKER FOR BREAST CANCER

Jihua Hao and James Basilion
Case Western Reserve University

Cysteine-rich intestinal protein one (CRIP1) has been identified as a novel pharmacological target for early detection, prevention, diagnosis, therapies, and treatment to breast cancer. The mRNA for this target is overexpressed 8–10-fold in approximately 90% of human breast cancers in both DCIS and invasive breast cancer by quantitative RT-PCR from analysis tissue bank of breast cancer and matched normal breast tissue (n=63), and in situ hybridization demonstrates close association of the expression with the ductal carcinoma cells. These data strongly support the development of imaging probes targeting CRIP1 to improve cancer detection.

Despite the potential utility of CRIP1 as an imaging target, significant efforts to develop CRIP1-specific ligands have not been attempted. Here we utilize phage display techniques to identify peptide ligands for purified human CRIP1. The consensus sequence motifs recognized by different domains on CRIP1 will be identified. The binding affinity of the peptides against CRIP1 will be determined by saturation binding experiments.

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P21-17: THE MAMMARY GLAND SEROTONIN SYSTEM AS A NOVEL BIOMARKER FOR BREAST CANCER

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Pregnancy-associated breast cancers have the worst prognosis; however, early full-term pregnancy is protective against breast cancer. The factors responsible for these paradoxical associations between pregnancy and breast cancer are unknown. Hence, it is very important to know and understand the systems that are present in the mammary epithelial cells but are altered in transformed cells and regulate their physiology. Previously our lab has demonstrated the presence of an intrinsic serotonin (5-HT) system in the mammary epithelial cells. Breast serotonin functions in an autocrine-paracrine manner to regulate mammary gland homeostasis and the lactation-involution switch. Other studies have shown that the involution microenvironment is promotional for breast cancer migration and metastasis. Hence, in this study we explored the 5-HT system in breast cancer cells in comparison with untransformed mammary epithelial cells. At a descriptive level, tryptophan hydroxylase 1 (TPH1), the rate-limiting enzyme for serotonin biosynthesis, is significantly induced at both the mRNA and protein levels in breast cancer cells, implying a high serotonin biosynthetic capacity in transformed breast cells. The serotonin reuptake transporter (SERT) is expressed at similar levels in normal and transformed breast cells, and type 7 serotonin receptor expression (5-HTR7) is induced in breast cancer cells. At the functional level, the signaling downstream of the 5-HT receptor is profoundly altered in breast cancer cells. The induction by serotonin of both c-AMP and p38 MAP kinase activity was altered in breast cancer cells. Serotonin is growth inhibitory for normal mammary epithelial cells, but breast cancer cells are refractory to growth inhibition by serotonin. Thus, the mammary serotonin system is a novel biomarker for breast cancer, which warrants further investigation as to its role in breast cancer physiology and its potential as a therapeutic target.

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P21-18: COMPUTATIONAL WORKFLOW DEVELOPMENT FOR THE CLINICAL APPLICATION OF PROTEOMIC PROFILING OF PLASMA SAMPLES

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Clinical applications of cancer proteomics include disease marker discovery for diagnosis, prognosis, and drug response as well as characterization of signaling and protein pathways. Due to the large dynamic range over which proteins must be detected in plasma (10¹⁰), several techniques for identifying differentially expressed proteins have to be combined, such as liquid chromatography followed by mass spectrometry and multiplex antibody arrays. High-throughput technology such as mass spectrometry requires large sample sizes, and the sheer volume of data collected during LC-MS/MS analysis of plasma samples (1.4 GB) requires that the workflow for data analysis is automated.

One of the major challenges of global proteomics is the lack of rigorous mathematical and statistical tools for analysis of data. While some techniques were developed in genomic studies for microarrays, they cannot be applied directly. For example, in microarrays, one immediately has the intensities for an object of interest, for example, genes, while in the MS/MS proteomics, the quantitative information is obtained for peptides and has to be combined into proteins.

The clinical global proteomics experiment is typically comprised of the following steps:

1. *Design of Experiment:* A mass spectrometry experiment should be carefully designed to include numerous factors in randomization and stratification scheme.
2. *Collection of Samples*
3. *Preparation of Samples:* To increase the sensitivity and to be able to detect the lower abundant proteins, high-abundance proteins are depleted prior to tryptic digestion.
4. *LC-MS/MS:* The analysis of tryptic peptides is performed by Thermo-Finnigan linear ion-trap mass spectrometer at Monarch Life Sciences.
5. *Identification and Quantification of Peptides:* The obtained spectra are searched over public databases, and peptides are quantified.
6. *Quantification of Proteins:* Since the database search does not provide the definite answer for similar proteins, such as in the case of polymorphic protein variances, we combine peptide-level quantitative information into protein families on the gene level.

7. *Biostatistical Analysis*: Peptide abundances are analyzed using the empirical Bayes approach. The overall significance of multiple hypothesis testing is controlled by the false discovery rate approach. The technique similar to the gene set enrichment analysis in microarrays is used to find statistically significant differences between the protein families. We account for biological dependencies between the proteins by annotating data with functions from GeneOntology and pathways from Ingenuity Systems.

The developed workflow can be applied to any disease. We tested it first on a dataset obtained from plasma samples of patients with cardiovascular disease (CVD). Since CVD is one of the most studied diseases, this dataset presented a great opportunity to validate the results by comparing it with known clinical markers from the literature. The clinical trial for breast cancer is still in the stage of sample collection, and we plan further to refine our workflow and use it on breast cancer samples.

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STEM CELLS I

Poster Session P22

P22-1: HUMAN MAMMARY STEM CELLS DETECTED USING A XENOTRANSPLANT MODEL REPRESENT A NOVEL POPULATION

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¹British Columbia Cancer Agency, ²University of Cambridge, and ³University of British Columbia

The mammary epithelium in normal adult female mice contains undifferentiated stem cells with extensive in vivo regenerative and self-renewal potential. Analogous cells presumably exist in the mammary glands of adult women but a specific and quantitative assay to characterize these cells and to delineate the pathways that regulate their function has yet to be developed.

We previously reported that histologically normal human mammary structures are produced in collagen gels containing dissociated cell suspensions prepared from normal reduction mammaplasty samples that are then placed under the renal capsule of highly immunodeficient, hormone-treated mice. We now show that such structures are reproducibly obtained from small inocula of suspended mammary epithelial cells after removal of contaminating hematopoietic and endothelial cells. We have also found that the structures generated contain mammary progenitor cells able to form adherent luminal, myoepithelial or bi-lineage colonies in vitro, as well as transplantable daughter "stem cells" able to produce similar progenitor-containing structures in secondary recipients, indicative of a regenerative process that recreates the normal developmental hierarchy. Mammary stem cells are detected at a frequency of 1 in $\sim 10^3$ – 10^5 cells in normal adult female breast tissue. Transplants of FACS-separated sub-populations show that these stem cells have a CD49^{high} EpCAM^{low} phenotype.

These findings set the stage for further biological and molecular characterization studies of normal human mammary stem cells and their relationship to human breast cancer stem cells.

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P22-2: DCIS STEM CELLS: INVESTIGATING THE ORIGIN OF THE INVASIVE PHENOTYPE

Lance Allen Liotta, Kirsten Edmiston, Virginia Espina, Julia Wulfschuh, Rosa Gallagher, Barbara Merritt, and Emanuel Petricoin
 George Mason University

We are testing the hypothesis that DCIS neoplastic cells already possess the full capability to invade and perhaps metastasize but that this malignant potential is delayed or held in check because the DCIS cells are confined in the unique niche microenvironment of the duct.

Specific Aims: (1) To microdissect in vitro living human DCIS structures consisting of a duct wall segment and its contained DCIS cells. Novel technology is being created to conduct living tissue laser microdissection. (2) To test the malignant phenotype of the harvested DCIS within immunodeficient SCID mice. (3) To map the proteomic signal pathway profile of the microdissected human DCIS compared to matched invasive, normal, hyperplasia, and benign-appearing breast tissue. (4) To derive continuous lines of DCIS stem cells. DCIS isolated lesions are stained with a vital stain, laser microdissected, and transplanted in a xenograft animal model. Signal pathway proteins and gene transcripts are analyzed in the microdissected breast lesions. The in vivo invasive and metastatic phenotypes are measured.

Results to Date: The Inova Fairfax procurement of human breast tissue has yielded 10 DCIS, 16 invasive, 29 stroma, 8 hyperplasia, and 5 normal tissue samples. We have developed vital staining chemistries that will highlight the living breast ducts in the fresh tissue so that under live tissue microdissection the DCIS lesions can be dissected under live tissue inverted microscopic view by the pathologist. It is too early in the project time line to report the success rate for generating invasive tumors from putative DCIS stem cells derived from DCIS organoids. These data will be presented at the time of the meeting. We have applied phosphoprotein microarray signal pathway analysis (ninety-one end points) and gene transcript profiling to microdissected human DCIS, compared to ductal hyperplasia, stroma, or invasive carcinoma in the same patient. The profile of signal pathway activation and gene transcript profiling in DCIS is most similar to the invasive cancer in the same patient. These data to date support the conclusion that the differentiated and metastatic potential of an individual patient's breast cancer is predetermined at the level of DCIS. The demonstration that DCIS cells already possess the capacity to invade, and perhaps metastasize, can profoundly alter future strategies for cancer screening and treatment. It will be important to identify the molecular and tissue structural factors that keep the DCIS cancer stem cells in check. The availability of continuous lines of human DCIS stem cells can become the foundation for a whole field of mechanistic and therapeutic studies.

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P22-3: INVESTIGATION OF STEM AND PROGENITOR SUBPOPULATIONS IN HUMAN BREAST TISSUE FROM BRCA1 AND BRCA2 CARRIERS

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Background and Objectives: We have previously isolated discrete populations of mouse mammary cells on the basis of cell-surface markers and defined a population that expresses "basal" markers and is highly enriched for mammary stem cells. Expanded stem cell numbers were noted in the preneoplastic phase in the MMTV-*wnt-1* mammary tumor model, consistent with tumors originating in stem cells in this model.¹ Interestingly, the stem cell-enriched basal population was "triple negative" for ER, PR, and ErbB2 expression. This phenotype is reminiscent of the basal subtype of breast cancer, suggesting that the mammary stem cell may be the "cell of origin" for this poor prognosis subset.² Since tumors that arise in women carrying a *BRCA1* gene mutation often exhibit a "basal" phenotype, we hypothesized that the breast stem cell pool is aberrant in breast tissue of *BRCA1* (and possibly *BRCA2*) carriers and that it becomes progressively and distinctively expanded in older carriers. Our objective was to evaluate putative breast epithelial stem cell markers in breast tissue from *BRCA* carriers and noncarriers enrolled in the Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (KConFab). Epithelial subpopulations were to be evaluated by establishing an in vitro culture assay and in vivo xenograft model.

Methods and Results: Our DOD-funded pilot project helped initiate the project using archival breast tissue samples. Subsequently, we have refined our approach, using fresh breast tissue prospectively from *BRCA* carriers and noncarriers (obtained from KConFab and the Royal Melbourne Hospital Tissue Bank). Using flow cytometric analysis and cell sorting, viable "lineage minus" (Lin⁻) cell suspensions were prepared by removing hematopoietic (CD45⁺) and endothelial (CD31⁺) cells. Lin⁻ cells were fractionated using different markers, revealing distinct subpopulations that include myoepithelial and luminal cells. Using an in vitro, three-dimensional culture assay, two subpopulations were defined that gave rise to distinct colonies comprising either small, homogenous, spherical structures or heterogeneous structures with occasional tubular outgrowths. Immunostaining suggested that they were derived from luminal and myoepithelial progenitors, respectively. Intriguingly, a Lin⁻ subpopulation from *BRCA1* carriers exhibited perturbed growth properties when compared to wild-type controls. Subpopulations have also been transplanted into the cleared and "humanized" mammary fat pads of NOD/SCID mice. Preliminary findings using non-*BRCA* tissue has identified a stem/progenitor population that preferentially yields breast outgrowths.

Conclusions: Our studies provide evidence for the existence of an epithelial cell hierarchy within human breast tissue. The perturbation noted in preneoplastic tissue derived from *BRCA1* carriers is currently under further investigation.

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P22-4: BAD SEEDS PRODUCE BAD CROPS: SIGNS OF A SINGLE STEP PROCESS OF BREAST TUMOR PROGRESSION

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Breast tumor progression is believed to be a multistep process, progressing sequentially from normal to hyperplastic, to in situ, and to invasive stages. Progression from an in situ to invasive stage is believed to be triggered by the overproduction of proteolytic enzymes mainly by tumor cells. These theories are supported by data from studies in cell cultures and animal models, while are hard to reconcile with two critical facts: [1] a subset of normal breast tissues shares a very similar genetic and biochemical profile with malignant lesions; [2] a vast majority of in situ tumors express high levels of proteolytic enzymes, but only 10%–30% of untreated in situ tumors progress to invasion during patients' lifetime.

As the myoepithelial (ME) cell layer is the sole source of several tumor suppressors, and degradation of the ME cell layer is the most distinct sign associated with invasive breast lesions, our recent studies have attempted to identify pre-invasive lesions with focal ME cell layer alterations. Using a double immunohistochemical technique with a panel of ME cell phenotypic and invasion-associated markers, our studies showed that about 15% of pre-invasive lesions contained normal appearing duct clusters in which each of the ducts harbored focal ME cell layer disruptions. In H&E stained

sections under low magnification, these clusters were often morphologically indistinguishable from clear-cut normal duct clusters. In immunostained sections, however, these duct clusters displayed several unique alterations: [1] a significantly higher rate of proliferation and genetic instabilities; [2] elevated expression of p53, c-erbB2, and other malignancy-signature markers; [3] aberrant expression of cell adhesion molecules; [4] a total loss of estrogen receptor (ER) expression in cells overlying focally disrupted ME cell layers, and some of these ER negative cells were arranged as tongue-like projections "puncturing" deep into the stroma or vascular-like structures that contained red blood cells.

Based on these and other findings, we have proposed that these normal appearing duct clusters are derived from a monoclonal proliferation of progenitor or stem cells that harbor defects in apoptosis- or cell cycle control-related genes, which confer upon these clusters growth advantages to continuously proliferate and to generate their own vascular structures. Therefore, these normal appearing ductal cells could progress directly to invasive or metastatic lesions without phenotypic changes. Progression may occur in two forms: [1] After multiple passages of cell divisions and differentiation, all the ME cells in these clusters become degenerated or disappeared, and the EP cells of each duct transform directly into a cluster of invasive cancer cells; [2] ER negative cell clusters overlying focally disrupted ME cell layers represent a less differentiated population of stem or progenitor cells, which retains the potential for multi-lineage differentiation. Thus, these ER negative cell clusters may constantly proliferate, and pump cells into blood vessels and lymphatic ducts, contributing to recurrent or metastatic lesions.

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P22-5: A NEW PARADIGM FOR AFRICAN AMERICAN BREAST CANCER INVOLVING STEM CELL DIFFERENTIATION IN A BREAST TISSUE ENGINEERING SYSTEM

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African American (AA) women have a higher incidence of breast cancer (BC) than white women before age 40 while the opposite is true after age 40. AA women are more likely to die from BC at every age. Although socioeconomic factors have in the past been attributed to explain these differences, data exist to support the less widely held hypothesis that there may be intrinsic biological differences in AA breast tissue compared with white breast tissue. Evidence for this hypothesis includes the fact that triple-negative breast tumors are more prevalent in AA women than in non-Hispanic white women although neither population is monolithic in its breast cancer phenotypes. Our laboratory has developed a novel tissue engineering system for human mammary epithelial cells (HMEC), both normal and malignant. This system allows for long-term establishment of nondiseased primary cultures that begin as three-dimensional attached mammospheres that are structures made up of 40–100 epithelial cells. These nondiseased mammospheres subsequently differentiate into functional, organotypic branching ducts and lobules that demonstrate ESA and CK -18, -19 staining, lumen, polarized nuclei, desmosomes, microvilli on apical surfaces and casein secretion. We hypothesize that intrinsic biological differences exist between AA and white breast tissue that can be demonstrated by the ability of this tissue to differentiate into a ductal network. We further hypothesize that this difference is due to a difference in the relative proportions of stem cells present.

We have established primary HMEC cultures from 36/36 breast reduction mammaplasty tissues, a success rate that is markedly higher than the rate shown in the literature (less than 2%). Nine of 36 of the subjects from whom this tissue came were AA and matched in socioeconomic status with the white women. We have found: (1) The more children a woman had, the less likely her breast tissue was to form ductal structures in vitro. This finding is consistent with the idea that lactational differentiation decreases the number of pluripotent stem cells in the breast and that stem cells provide the capacity to form ductal structures in our system. (2) Premenopausal breast tissue was more likely to form ductal structures than postmenopausal tissue. (3) Race contributed as a modifying factor in the ability to form ductal architecture in culture with socioeconomically matched AA tissue demonstrating more of an ability to spontaneously differentiate than white tissue. (4) Preliminary stem cell analysis using flow cytometry with markers identified below is consistent with a higher proportion of stem cells in cell lines derived from AA breast tissue relative to tissue from white women.

Time-lapse imaging was used to collect data on cell behavior. Putative mammary stem cells were analyzed using flow cytometry using ESA+, MUC-, $\alpha 6$ integrin+ antibodies as well as with cloning. Stem cell proportion will be correlated with the capacity for differentiation in each tissue. These findings are consistent with the idea that greater proportions of stem cells may exist in the AA population than in age- and

parity-matched white women. If this hypothesis were true, then one might expect a higher mortality in AA women because of the presence of a stem cell population more likely to form an aggressive tumor type.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0581 and Susan G. Komen for the Cure.

P22-6: ADULT HUMAN MESENCHYMAL STEM CELLS ENHANCE BREAST CANCER TUMORIGENESIS AND PROMOTE HORMONE INDEPENDENCE

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Adult human mesenchymal stem cells have been shown to home to sites of breast cancer and to integrate into the tumor stroma. While this has been described to influence breast cancer metastasis, here we demonstrated the effect of adult human mesenchymal stem cells on primary tumor growth and the progression of breast tumors to hormone independence. Bone marrow-derived mesenchymal stem cells mixed with the estrogen receptor-positive, hormone-dependent breast carcinoma cell line MCF-7 enhance primary tumor growth as well as promote hormone-independent tumor growth of subcutaneous injections in immunocompromised mice. Our studies have also shown an increased expression of the chemokine stromal derived factor 1 (SDF-1) in these mixed cell tumors as compared to the MCF-7 only tumors. We hypothesize that it is this increase in SDF-1 provided by MSCs that acts in a paracrine fashion on the breast cancer cells, which leads to a shift to hormone-independent growth while still remaining estrogen sensitive. Taken together, our data reveal the relationship between tumor microenvironment and tumor growth and progression to hormone independence through the release of stromal factors. This tumor stroma-cell interaction may provide a novel target for the treatment of hormone-independent, endocrine-resistant breast carcinoma.

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P22-7: Wnt-INDUCED PROGENITORS: ARE THEY HIGHLY MUTABLE?

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Background: Ectopic Wnt signaling is highly tumorigenic for mammalian epithelia. We have previously found that Wnt effectors induce mouse mammary stem cells to accumulate and that this is closely correlated with the risk of subsequent tumor development (Liu et al., 2004). These somatic stem cells are a likely source of tumor precursor cells; they have the longevity that is required to fix and accumulate the genetic changes that confer a growth advantage on preneoplastic cells. In an effort to describe why increased stem cell number leads to tumor development, we proposed to test whether Wnt-induced mammary stem cells were more mutable than normal stem cells. In order to do this, we aimed to transfer normal and Wnt-induced mammary epithelial cell populations to suspension culture, to treat them with mutagenic carcinogens, and to evaluate the fraction of mutant mammospheres (clonogens) that developed. Our hypothesis was that Wnt effectors change the predominant type of stem cell division from asymmetric self-renewal (associated with little genomic risk) to one that creates a population of long-lived but highly mutable stem cells that are likely to acquire oncogenic mutations.

Methods: After dissociation of primary MECs from normal breast tissue, they were transferred to suspension culture to allow anchorage-independent cells to expand into mammospheres. The rate of mutation for Wnt-induced compared to normal stem cells was estimated using selection for HPRT activity.

Results: We found that the number of mammospheres that develop in Wnt-induced mammary epithelial populations was reduced by 90%. This was surprising to us because we have already established that the stem cell activity of these populations is increased in vivo (using fat pad transfer) and that a subpopulation enriched in stem cells (SP fraction) is also increased (in $\delta N\beta$ cat MECs by a factor of 15). This result precluded us from measuring mutation rates as proposed.

Conclusion: The use of murine mammosphere cultures as a surrogate for evaluating stem cell number and activity was thrown into doubt by our results. Indeed, mammospheres have never been shown to have significant stem cell activity by us or other groups.

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P22-8: SMALL MOLECULE COMBINATORIAL LIBRARIES TO IDENTIFY NEW MAMMARY STEM CELL MARKERS

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Although specific markers for adult stem cells have been described for several years, a number of those are not specific for a putative cell type or for stage of differentiation. Identification of new or novel cell surface or cytoplasmic markers would be extremely useful in the identification of stem cells from normal and malignant tissues. To identify new surface markers, we used combinatorial libraries to screen for novel peptides capable of surface binding to stem cells. The libraries were produced by split-mix synthesis of amino acids on polystyrene beads. For that, 8-mers with D-cysteine residues at both ends were created to stabilize the peptide and resist proteolysis. Large spatially separable but non-addressable chemical libraries on beads were identified by sequencing a coding region. Although stem cells from mammary tumors have been described for several years, there remains a very large interest in specifically identifying those cells. Recently, the neural stem cell marker CD24 (heat-stable antigen) and the skin stem cell marker CD29 (β1-integrin) were used to enrich a population of lineage (TERT119⁺, CD45⁺, CD31⁺) mammary cells from the stem cell population. Those cell surface markers are neither new nor restricted to the putative mammary stem cell. Our goal was to identify novel markers on stem cells from normal, pre-cancer, and malignant mammary tissues. Putative stem cells from normal mammary, mammary pre-cancer, and mammary tumor tissues were isolated by fluorescence cell sorting. The resultant stem cell-enriched (SCE) and stem cell-depleted (SCD) populations were identified with different lipophilic fluorescent dyes then incubated together with beads, each conjugated with their own unique peptide. Only beads positive for cells from the SCE population were selected. Those peptides were then used to search for putative stem cell binding sites and possible novel markers. Analysis of those sequences indicated some standard and some unique motifs recognized. Commonly occurring sequences were used to design more specific peptides and can be incorporated to isolate stem cells from a mixed cell population. Not only can the new markers be used to detect stem cells, but they can be used as a means for possibly differentiating between normal, pre-cancer, and tumor stem cells.

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P22-9: GENE EXPRESSION CHARACTERISTICS OF SIDE POPULATION CELLS FROM BREAST CANCER

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Cancer stem cells represent a new paradigm in tumor biology. Toward the goal of breast cancer stem cell purification and characterization, we have isolated putative breast cancer stem cells based on dye efflux after Hoechst 33342 staining. Alternative approaches for stem cell purification with other fluorescent dyes and flow cytometric sorting (FACS) have also been tested. Using the human bone-metastatic MDA-MET breast cancer cell line (a derivative of MDA-MB231), cells were stained with the standard Hoechst 33342 method and compared with doxorubicin/BODIPY-vinblastine, rhodamine 123, mitoxantrone, and BODIPY-prazosin. Cells were gated by FACS into high-fluorescence "Main Population" (MP) and low-fluorescence "Side Population" (SP) cells. The SP ranges in abundance from 0.1% to 2%, depending on the dye. The SP cell gate using Hoechst 33342 includes ~0.3% of the total live cells, 80% of which are true SP cells based on clearing of the gate by verapamil inhibition of the ABCG2 transporter. To characterize the stem cell properties of the SP cells, we tested for enrichment of gene expression, using quantitative RT-PCR, expressing data as the SP/MP ratio after normalization to a housekeeping gene. The initial focus was on 4 genes: two ABC membrane efflux pumps that confer drug resistance, ABCG2/BCRP (breast cancer resistance protein) and ABCB4/MDR3 (multidrug resistance 3), and two stem cell transcription factors that confer self-renewal, Oct 4 and Nanog. Two different protocols (Hoechst 33342 or doxorubicin/BODIPY-vinblastine) showed comparable SP/MP expression ratios of ~20–100 for ABCG2 and ABCB4/MDR3. Expression of both Oct 4 and Nanog was also upregulated in the SP after Hoechst 33342 sorting (SP/MP ~70–90), but these ratios for Oct 4 and Nanog were dramatically increased to >1000 with the doxorubicin/BODIPY-vinblastine method. Rhodamine 123 and BODIPY-prazosin failed to yield SP cells with significant enrichment of these genes. Comparative drug cytotoxicity of SP versus MP cells was measured in graded concentrations (0–100 μM) of doxorubicin, cyclophosphamide, and methotrexate; however, no significant differences in survival between SP and MP cells were observed. Sorted SP cells, although showing higher pump expression at the time of sorting, appear to quickly lose this characteristic that would confer drug tolerance. After *in vitro* culture of isolated SP cells, a second round of Hoechst 33342 sorting reveals poor preservation of the SP phenotype, and reappearance of a predominant MP population. We used a novel transcriptome analysis program (GEDI; Eichler et al., *Bioinformatics* 2003; 19:2321) that analyzes microarray datasets in a "sample oriented" rather than "gene oriented" manner, in keeping with a systems biology approach. This was applied to Hoechst 33342 SP and MP popula-

tions and corrected for effects of cytotoxicity and sorting by comparison with Hoechst 33342-treated but unsorted cells, and control unsorted cells. Using a static analysis option and Pearson correlation coefficient for gene clustering, a gene cluster elevated in SP over MP includes MIF (macrophage migration inhibitory factor; known to be upregulated in breast cancer) and other genes not yet linked to cancer.

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P22-10: USE OF HYALURONAN NANOPARTICLES TO DETECT BREAST TUMOR PROGENITOR CELLS

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Background: CD44⁺/CD24^{low}/ESA⁺ breast tumor "progenitor" or "initiating" subsets represent <3% of primary tumor cells but are more tumorigenic than other tumor phenotypes and give rise to a tumor xenograft that resembles the original primary tumor in its phenotypic heterogeneity (Al-Hajj et al. 2003). The gene signature of these cell subsets, in particular those that are enriched for CD44 expression, predict poor clinical outcome (Liu et al. 2007; Shipitsin et al. 2007). Several immortalized human breast cancer cell lines express this phenotype including MDA-MB-231 (Sheridan et al. 2006). MDA-MB-231 breast tumor cells express not only high levels of CD44 but also the cell surface form of RHAMM (HMMR), which like CD44 is a hyaluronan (HA) receptor. We predict tumor progenitor cells will endocytose HA more rapidly than non-progenitor cells and this property can be used to selectively image tumor progenitor subpopulations.

Purpose: Despite advances in pre- and intra-operative imaging, the detection of early breast cancer lesions has remained as an issue due to low sensitivity of existing modalities.

Results: We developed methods for labeling HA fragments (M.W.10 kDa) with fluorochromes and heavy metals. We have demonstrated that uptake of tagged-HA is significantly greater in MDA-MB-231 than in MC7 breast tumor cell lines. We quantified the half-life of plasma HA following I.V. and subcutaneous injection or oral gavage of these HA imaging agents. I.V. injection resulted in the shortest plasma half-life (T1/2=12 hr) but the highest plasma levels of tagged-HA and was chosen as the most ideal route of administration. Two to three mm tumors of MDA-MB-231 cells were grown as xenografts in immune compromised rats. Gadolinium-HA fragments accumulated in detectable levels within the tumor xenografts and levels were significantly higher in tumors than other tissues excepting the liver.

Conclusions: These experiments provide confirmation for "proof-of concept."

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P22-11: IDENTIFICATION OF MICRORNAS INVOLVED IN NORMAL MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

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The mammary gland's capacity to regenerate during multiple rounds of pregnancy, lactation, and involution has been proposed to be due to the presence of a population of self-renewing mammary gland stem cells. Our studies are based on the hypothesis that miRNAs play an important role in normal mammary gland development, mammary gland stem cell maintenance and self-renewal and, when aberrantly expressed, may participate in the transformation of a lineage-restricted mammary gland stem cell to a cancer stem cell. We are interested in identifying miRNA genes that play essential roles in mammary gland development and breast cancer with emphasis on their role in normal and cancer stem cells. Identification of miRNAs that are critical modulators of normal mammary stem cells will help give insight into the changes that may regulate this transformation.

As a model for mammary gland development, we use a mouse mammary epithelial cell line, COMMA-D β geo. This cell line has two subpopulations of cells, indicated by the presence of the cell surface marker stem cell antigen-1 (Sca-1), displaying different morphogenic potentials. The Sca-1+ cells have progenitor-like activity in both in vivo and in vitro assays compared to Sca-1- cells. miRNA arrays were used to generate the miRNA profiles of these two populations to determine differentially expressed miRNAs. From these arrays, we have identified a panel of miRNAs that we are currently examining in both in vivo and in vitro assays for involvement in normal mammary gland development and miRNAs that may become aberrantly expressed in breast cancer.

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P22-12: ARE BREAST TUMOR STEM CELLS RESPONSIBLE FOR METASTASIS AND ANGIOGENESIS?

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The current dogma of metastasis is that most primary tumor cells have low metastatic potential, but rare cells, less than one in ten million, within large primary tumors acquire metastatic capacity through somatic mutation. A recent report has demonstrated the importance of tumor stem cells in breast cancer development. Based on this information, we propose an alternate model of metastasis and hypothesize that the breast tumor stem cells are the subpopulation of cells that are present in the heterogeneous primary breast tumor and possess the unique properties of an angiogenic and metastatic phenotype. In this study, we used Her2/neu transgenic mice as our model system. Murine breast tumor stem cells were enriched from Her2/neu transgenic mammary tumors using two cell surface markers, CD44 and CD24. In vitro, CD44+/CD24- (enriched breast tumor stem cell population) cells secreted similar amounts of VEGF than CD44-/CD24+ and unsorted Her2/neu tumor cells. In addition, cell invasion was similar between CD44+/CD24- cells, CD44-/CD24+ cells, and unsorted Her2/neu tumor cells. Next, we assessed the metastatic potential of CD44+/CD24- cells using the pulmonary colonization metastasis model. We injected unsorted Her2/neu tumor cells, sorted CD44+/CD24-, or sorted CD44-/CD24+ cells in the tail veins of athymic nude mice. After 8 weeks, mice were euthanized and lungs were resected for analysis. Lung sections from animals in all three groups were absent of tumor cells indicating that the injected cells did not metastasize to the lungs. Our negative results could be due to two reasons: the use of CD44 and CD24 may not be sufficient to adequately enrich for putative breast tumor stem cells in the Her2/neu transgenic mouse model, and the athymic nude mice used in this study may not be immunocompromised enough for tumor cells to establish and grow. Our future plan is to continue this work and identify a better set of cell surface markers to enrich for putative breast tumor stem cells from Her2/neu transgenic mice.

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P22-13: MAMMARY STEM CELLS AND RESIDUAL NEOPLASTIC DISEASE

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A cardinal feature of human breast cancers is the survival and persistence of residual neoplastic cells in a presumed quiescent state following the apparently successful treatment of the initial tumor. Ultimately, these residual cells re-emerge from their dormant state and resume growth, leading to cancer recurrence. The ability of residual neoplastic cells to survive for many years has raised the possibility that these cells may share important properties with mammary stem cells. Since the ability to self-renew is common to both normal stem cells and cancer cells and since several signaling pathways implicated in cancer have also been implicated in stem cell self-renewal, it has further been proposed that normal stem cells may represent a preferred target for transformation. To better define the molecular and cellular events that contribute to breast cancer recurrence, we have developed a series of doxycycline-inducible transgenic mouse models that display many features of human breast cancer progression, including metastasis, residual neoplastic disease, and recurrence. Following induction with doxycycline, bistransgenic animals develop invasive mammary adenocarcinomas. Subsequently, the majority of these tumors regress to a clinically undetectable state following doxycycline withdrawal and oncogene downregulation. However, many fully regressed tumors spontaneously recur in the absence of oncogene expression over periods of up to a year, ultimately resulting in the death of the animal.

We hypothesize that the population of residual neoplastic cells that survive oncogene downregulation and persist in the mammary gland following tumor regression are enriched for cells that share critical features with mammary stem cells and/or cancer stem cells. The experiments supported by this proposal attempt to determine the relationship between residual neoplastic cells, stem cells, and cancer stem cells using functional tests and to thereby analyze properties of residual neoplastic cells that are relevant to tumor recurrence. Initial experiments are aimed at determining whether cells with tumorigenic potential are enriched in residual neoplastic lesions. These are complemented by studies to determine whether cells bearing markers for mammary stem cells are enriched in residual lesions and whether residual neoplastic cells possess functional properties of mammary stem cells. Finally, we are attempting to determine whether mammary stem cells constitute preferential targets for oncogene-induced transformation. Tumor recurrence is responsible for the vast majority of breast cancer deaths. Studying residual neoplastic cells to determine their potential relationship to mammary stem cells or cancer stem cells may shed light on the role of these cells in breast cancer progression and may provide new ways to prospectively identify tumors posing greater risk for recurrence. This approach should facilitate the development of more appropriate therapies targeted against this critical subpopulation of tumor cells.

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P22-14: Wnt SIGNALING IN MAMMARY STEM CELL DEVELOPMENT AND CANCER

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Background and Objective: A recent study described that a functional mammary gland can be generated from a single cell, suggesting the existence of mammary stem cells (MaSCs). Numerous reports also have repeatedly confirmed that reproductive history represents the strongest risk/protective factor for breast cancer. The recently identified parity-induced mammary epithelial progenitor (PI-MEP) cells, displaying stem cell characteristics, may contribute to differences in cellular composition between the nulliparous and parous glands. Wnt signals control normal mammary development at various phases. Several Wnt signaling molecules have been linked to neoplastic transformation of the mammary gland. We have demonstrated previously that the Axin tumor suppressor negatively regulates a Wnt signal essential for lobulo-alveolar development in late pregnancy. Axin apparently exerts its effects through modulating the stability of β -catenin. We hypothesize that MaSCs play a key role in breast tumorigenesis and that this stem cell population is altered during parity. While nulliparous glands consist of pluripotent MaSCs, PI-MEP changes the precursor cell population and property in parous glands. The primary focus of our study is to explore the role of Wnt signaling in regulating MaSCs and PI-MEP cells during mammary development and their involvement in tumorigenesis.

Methodologies: We have created an advanced system for conditional gene expression in mice by integrating the Cre-mediated and tetracycline-dependent expression systems to investigate PI-MEP in breast development and cancer. This novel approach is uniquely suited for manipulating gene activity/cellular signaling in the PI-MEP cells. We will first examine if the stem cell population and characteristics are altered by parity. Second, we will determine if Wnt signaling affects the population of MaSCs and PI-MEP. Finally, we will examine if eliminating the stem cell populations alleviates cancer development in a transgenic mouse model.

Results to Date: We found that Wnt signaling increases the population of MaSCs in nulliparous glands. The parity-mediated remodeling process also affects this stem cell population. Using our novel transgenic expression system to inhibit Wnt signaling by manipulating the β -catenin/Axin regulatory circuit, the PI-MEP population is apparently altered in parous glands.

Conclusion: The MaSCs and PI-MEP cells are regulated at different phases of mammary development. Wnt signaling is intimately involved in controlling the development of these precursor cell populations. The importance of stem cells in tumorigenesis provides an operative model to achieve breast cancer prevention and therapy in humans. Our study will provide an urgent need for information about the existence and identity of stem cells at various developmental stages. Understanding the differential property of these precursors in nulliparous and parous glands is critical for cancer susceptibility and resistance associated with parity.

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CELLULAR DEVELOPMENT

Poster Session P23

P23-1: UNMASKING STEM/PROGENITOR CELL PROPERTIES USING SHORT-TERM TRANSPLANTATION

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Background: Normal mammary epithelial stem cells are long-lived, self-renewing, and give rise to all progenitor and differentiated epithelial cell types. Stem cells, and progenitor cells derived from them, are also hypothesized to be a cell of origin for breast cancer. Thus, it is essential to define the full range of division-competent stem/progenitor cell types in the mammary gland and to understand their regulation for successful treatment of breast cancer.

Normal mammary stem cells are distributed throughout the ductal tree. Limiting dilution transplantation assays suggest stem cells represent about 1 in 100 to 1 in 2,000 cells. Coupled with electron microscopy (EM) studies showing only three division-competent epithelial cell types in the intact mouse mammary gland, these data support prevailing models in which division-competent stem/progenitor cells comprise only a minority of epithelial cells. In conflict with such models, other data suggest the true percentage of division-competent stem/progenitor cells in the mammary gland may be dramatically underestimated using common assays.

Rationale: Short term transplantation assays using small fragments of intact mammary duct transplanted into epithelium-free fat pads of host mice offers an excellent opportunity to test the prevailing stem/progenitor cell model, and to explore the developmental plasticity of mammary epithelium. With small fragments (~1000 cells), three-dimensional structure and cell-cell interactions are largely maintained, while the inhibitory effects of neighboring epithelium are minimized. Thus, stem/progenitor cells should be in an environment more favorable to cell division. In addition, onset of regeneration can be timed precisely.

If division-competent stem/progenitor cells represent a small percentage of all epithelial cells, the initial rate of cell division in transplanted fragments should be low, similar to that of dissociated cells, and limited to relatively undifferentiated epithelial cell types defined previously by EM. However, if stem/progenitor cells can include more differentiated, yet division-competent cells, the initial rate of cell division in fragments should be higher than that observed in transplants of dissociated cells, and include a broader range of epithelial cell types.

Methods and Results: Mammary epithelial fragments were harvested from mice expressing Enhanced Green Fluorescent Protein (EGFP) and transplanted contralaterally into epithelium-free fat pads of mature virgin mice. Transplanted glands were harvested at four hour intervals with mice injected with BrdU 6 hours before harvest to identify cells participating in gland regeneration. Harvested fragments were evaluated for stem/progenitor cell markers and cell division at each timepoint.

Transplanted fragments undergo a profound change in gene expression and behavior prior to the onset of proliferation. Expression of cell-cell junction markers and differentiation markers is reduced transiently and cells reorganize substantially. Once remodeling has occurred, expression of these markers returns and a near synchronous burst of proliferation begins.

Conclusions: Mammary epithelial cells are more plastic than generally recognized and a larger number of cells than generally recognized retain division competence.

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P23-2: LYMPHATIC DIFFERENTIATION OF ENDOTHELIAL PRECURSOR CELLS FOR A CELL-BASED LYMPHEDEMA THERAPY FOR BREAST CANCER PATIENTS

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Lymphedema, or a tissue swelling, in the arm is the most common post-operative complication of breast cancer patients. Studies show that 5% of breast cancer patients may develop various degrees of lymphedema in their first year of surgical operation and 20% in their lifetime. Although a recent employment of the sentinel lymph node biopsy technique tends to reduce the prevalence, this disfiguring complication still remains a considerable physical, psychological, and social burden to cancer survivors without any treatment available. The object of this study is to develop a technology that allows us to obtain lymphatic endothelial cells (LECs) from patient-derived adult stem cells to rebuild the damaged lymphatic vessels of breast cancer survivors with lymphedema. It has been demonstrated that LECs are derived from blood vascular endothelial cells (BECs) during embryonic development and that BECs isolated from human neonatal foreskin could be successfully reprogrammed to adopt LEC phenotypes in vitro. Moreover, it has been recently shown that EPCs can be isolated from human peripheral blood and that these cells can form functional blood vessels when transplanted into an animal model. Here, we present our data showing that the home-

odomain transcription factor Prox1 is essential for lymphatic differentiation of EPCs and for maintenance of lymphatic phenotypes.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0539.

P23-3: Cdc42 AND ITS REGULATORY PROTEINS CONTROL EPITHELIAL POLARITY AND TUMORIGENICITY IN 3-DIMENSIONAL CULTURE

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Background and Objectives: Epithelial cells in the mammary glands form structures whose basic unit is a polarized monolayer of cells arranged in a tubule or acinus around a lumen. These cells are highly susceptible to neoplastic transformation, which is characterized by the loss of polarity and organized tissue structure. This tissue architecture of normal epithelia can be recapitulated in a 3-dimensional (3D) culture matrix in vitro. In contrast to normal cells, cancer cells in 3D cultures fail to form these polarized cysts or acini, instead producing solid masses of cells with very small or no lumens. We proposed to take advantage of this characteristic to screen for mutations in human breast cancer cells that can restore normal acinar architecture.

Methodologies: Epithelial cells were cultured in 3D matrigel or collagen matrices. Transgenes or short hairpin RNAs were stably expressed, and their ability to revert tumorigenic phenotype was determined by confocal microscopy.

Results to Date: We have identified the Rho family GTPase Cdc42 and two proteins that regulate the Rho family of proteins as key molecules in this process. Inhibition of Cdc42 blocks the hyper-proliferation of transformed acini while activation causes partial reversal of polar architecture without blocking hyperproliferation, suggesting that a tight control of Cdc42 function is required for maintaining normal acini. Knocking down of the Cdc42 activator β -Pix also led to a loss of polarity. A knock-down of P190RhoGAP, which inhibits Rho, was also found to cause polarity loss. Both of the loss of polarity phenotypes could be rescued by growing these acini in natural basement membrane, Matrigel.

Conclusions and Potential Impact: Our studies show that acute functional inhibition of Cdc42 in tumorigenic cysts leads to a reduction in acinar size, indicating that Cdc42 is required for the hyperproliferation of cysts. Here we have identified two of the key genetic changes, viz., loss of β -Pix and P190RhoGAP, that when accompanied by the absence of a proper tissue microenvironment, lead to loss of polarity and a transformed phenotype. Cdc42 has been identified as a key target for breast cancer drug discovery. Our studies further elucidate the regulatory elements of the Cdc42 family of small GTPase pathway that control epithelial polarity and indicate that these may be additional targets for breast cancer drug discovery.

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P23-4: Mist1 IS CRITICAL TO MAINTAINING THE TERMINAL DIFFERENTIATED STATE OF MAMMARY ALVEOLAR CELLS

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The development of mammary glands relies on complicated signaling pathways that control cell proliferation, differentiation, and apoptotic events through transcriptional regulatory circuits. Alterations in any of these pathways can lead to neoplastic changes that may progress to metastatic disease. One key family of transcription factors utilized in mammary gland development is the helix-loop-helix/basic helix-loop-helix (HLH/bHLH) protein family. In this study, we sought to determine if Mist1, a tissue-restricted Class II bHLH transcription factor, is functional in mammary gland tissue. Analysis of mouse and human mammary glands revealed that Mist1 protein accumulated exclusively in secretory alveolar cells. Mist1 transcripts were also differentially expressed in mouse mammary epithelial Scp2 cells that were induced to differentiate by addition of lactogenic hormones. To determine the importance of Mist1 to mammary gland development and cell growth control, we next examined how loss of Mist1 protein affected mammary epithelial cells. Our studies showed that Mist1 null lactating mammary glands were defective in lobuloalveolar organization, exhibiting shedding of cells into the alveolus lumen and premature activation of the Stat3 signaling pathway. These cells also failed to assemble functional gap junctions, leading to altered cell growth properties that were due to decreased p21^{CIP1/WAF1} protein levels. Similar defects were also observed in Scp2 cells where Mist1 expression was decreased using a viral shRNA approach. Knock-down of Mist1 transcripts blocked cells from expressing terminal differentiation gene products, including β -casein and whey acidic protein. These findings suggest that loss of Mist1 alters the terminal differentiation state and growth arrest program of mammary epithelial cells, revealing for the first time the importance of a Mist1 transcriptional network to nor-

mal mammary gland growth control. Current studies are aimed at examining if *Mist1* prevents neoplastic changes in mammary epithelial cells upon oncogene activation.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0319.

P23-5: CONNECTIVE TISSUE GROWTH FACTOR ENHANCES MAMMARY EPITHELIAL CELL LACTOGENIC DIFFERENTIATION VIA INTEGRIN MEDIATED SIGNALING

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Mammary epithelial cells go through a series of developmental changes during pregnancy and lactation including proliferation, differentiation, secretion and apoptosis. HC11 mouse mammary epithelial cells, which undergo lactogen-induced differentiation in cell culture, were used to follow the changes in gene expression during this process. The expression profiles of over 20,000 genes were examined in HC11 cells undergoing lactogenic differentiation using DNA microarray analysis. The results indicate the involvement of numerous genes and pathways in the differentiation of mouse mammary epithelial cells in culture. Because the response of HC11 mammary epithelial cells to basement membrane and stromal protein-induced signals controls the degree of differentiation in this cell background, our analysis of the global expression profiling results focused on genes that encode proteins involved in cell adhesion. Significantly elevated expression of laminin $\alpha 5$, Rho kinase (ROCK), and Rac1 were detected. In addition, osteopontin and the small integrin binding protein connective tissue growth factor (CTGF/CCN2) were highly elevated. Validation of the gene expression pattern for more than thirty genes was performed in HC11 cells and in mouse mammary glands, revealing a pattern of expression during pregnancy and lactation that declined during involution of the glands. Hence, there are significant parallels in expression between HC11 cells undergoing differentiation and mouse mammary gland during late pregnancy and lactation.

The novel finding that the CTGF is highly elevated during lactogenic differentiation of HC11 mouse mammary epithelial cells and in mouse mammary gland during pregnancy and lactation suggested that it may play a role in the mammary epithelial cell differentiation. Further studies revealed that in HC11 cells CTGF is transcriptionally regulated by dexamethasone, and CTGF expression is not dependent on TGF β . CTGF contributes to and is required for lactogenic differentiation of HC11 cells, as demonstrated by increased differentiation following expression of plasmid-encoded CTGF and decreased differentiation following depletion of endogenous CTGF with siRNA. HC11 mouse mammary epithelial cells infected with an adenoviral vector encoding CTGF exhibit increased lactogenic differentiation and primary mammary epithelial cell cultures established from pregnant mice show enhanced transcription of β -casein following infection with CTGF-adenovirus. Using a CTGF Tet-off system in the HC11 cell background, the elevation of CTGF mediated enhancement of β -casein transcription, mammosphere formation, and stabilization of Stat5 phosphorylation, all of which are markers of lactogenic differentiation. CTGF also increased the level of $\beta 1$ integrin and the phosphorylation of downstream signaling mediators FAK and AKT. CTGF Tet-off HC11 cells display enhanced and sustained growth in serum-free conditions. Elevated CTGF expression in the Tet-off HC11 cells abrogated the extracellular matrix enhancement of lactogenic differentiation thus highlighting the role of CTGF in regulating integrin-dependent signaling. These results demonstrate that the production of stromal factors is an important component of differentiation in mammary epithelial cells and that the regulation of CTGF by glucocorticoids may play a critical role in this aspect of the control of mammary differentiation.

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P23-6: APPLICATION AND VALIDATION OF IN CELL WESTERN TECHNIQUES FOR ANALYSIS OF EXPRESSION IN MICROFLUIDIC CELL CULTURES

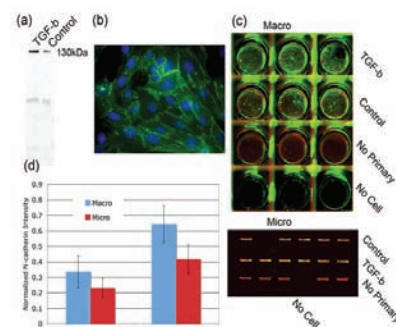
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Microfluidic methods for cellular studies are wide ranging from basic cell culture to 3-D tissue engineering. Cell culture can be performed in microfluidic devices with minimal cell numbers, typically with 250+ fold less total cell number in each channel than each well of a 6-well plate, with the same cell surface density. However, a principal issue that prevents microfluidic assays from being integrated as a tool in vitro assays is the lack of simple, quantitative readouts.

Immunostaining techniques such as western blotting are common practice in traditional biology laboratories, but the amount of cell lysate needed to perform a typical assay is 1,000- to 5,000-fold more than a microfluidic culture can typically provide (e.g., one well of a 6-well plate, or more). While the low cell numbers precludes the possibility of performing traditional western blots from microfluidic cultures, it means that doing a western type assay in situ becomes beneficial.

In this work, techniques for performing in cell westerns (ICWs) have been applied to a variety of analyses. To perform an ICW, cells are grown in monolayer cultures using typical tissue culture protocols then fixed and stained just as for immunocytochemistry using fluorescent secondary antibodies. Using a commercially available laser scanner, the total integrated intensity of a group of cells is determined and normalized to a loading control such as β -actin or DNA content. A quantitative measurement of the levels of the protein of interest per cell can be determined without the need to obtain and process sufficient amounts of cell lysate then perform the gel electrophoresis and blotting procedures for a traditional gel-based western.

Work to validate this technique for future work studying primary mammary stem cells in vitro has included performing ICWs with a well-documented cellular response (that of epithelial cells undergoing epithelial to mesenchymal transition (EMT) in response to transforming growth factor- β [TGF- β]). The figure shows the traditional western (a), immunocytochemistry (b), and ICW data (c and d) for a mammary epithelial cell line expressing N-cadherin after 48-hour TGF- β treatment. Work in progress includes studying stress responses in microfluidic cultures to examine the potential differences between macro- and micro-scale cultures to determine the suitability for stem cell culture.



Analyzing the expression of N-cadherin in mammary epithelial cells when treated with TGF- β using traditional Westerns (a), immunocytochemistry (b), and In Cell Westerns in both macro- and micro-scale cultures (c and d).

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P23-7: LOSS OF CYCLIN D1 IN CONCERT WITH DEREGLATED ESTROGEN RECEPTOR α EXPRESSION INDUCES DNA DAMAGE RESPONSE ACTIVATION AND INTERRUPTS MAMMARY GLAND MORPHOGENESIS

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We have previously shown that increased and deregulated estrogen receptor α expression in the mammary gland leads to the development of proliferative disease and cancer. To address the importance of cyclin D1 in ER α -mediated mammary tumorigenesis, we crossed ER α -overexpressing mice with cyclin D1 knockout mice. Mammary gland morphogenesis was completely interrupted in the ER α -overexpressing cyclin D1-deficient triple transgenic mice. In addition to a highly significant reduction in mammary epithelial cell proliferation, cyclin E was upregulated resulting in DNA damage checkpoint activation and apoptosis. This imbalance between proliferative and apoptotic rates in conjunction with remarkable structural defects and cellular disorganization in the terminal end buds interrupted ductal morphogenesis. Interestingly, the structure of the mammary fat pad was fundamentally altered by the consequences of overexpressing ER α in the epithelial cells in the absence of cyclin D1, illustrating how alterations in the epithelial compartment can impact surrounding stromal composition. Transplantation of embryonic ER α -overexpressing and cyclin D1-deficient mammary epithelium into the cleared fat pad of wild-type mice did not rescue the aberrant mammary gland phenotype, indicating that it was intrinsic to the mammary epithelial cells. In conclusion, although cyclin D1 is not essential for proliferation of normal mammary epithelial cells, ER α -overexpressing cells are absolutely dependent on cyclin D1 for proliferation. This differential requirement for cyclin D1 in normal versus abnormal mammary epithelial cells supports the application of cyclin D1 inhibitors as therapeutic interventions in ER α -overexpressing breast cancers.

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P23-8: FORMIN-CONTROLLED CORTICAL ACTIN DYNAMICS IN BREAST CANCER CELL MIGRATION THROUGH THREE-DIMENSIONAL MATRICES

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Mammalian diaphanous (mDia)-related formin Rho GTPase-effector proteins generate nonbranched actin filaments in cellular responses to proliferative, adhesive, and migratory stimuli. While formins contribute essential protrusive structures at the leading edge of migrating nontransformed cells, their contribution to metastatic pro-

cesses has not been fully evaluated. Recently, we discovered mDia-mediated actin assembly is modulated by the Dia-interacting protein (DIP). It was observed that expression of a conserved DIP leucine-rich C-terminus (LRR), which binds to and specifically inhibits the formin mDia2 *in vitro*, triggered continuous nonapoptotic membrane blebbing of the cell cortex. Plasma membrane blebbing occurs in several normal contexts, such as during amoeboid cell movement (ACM), and is dependent upon the tight control of the cortical actin cytoskeleton. ACM has been observed in metastatic breast cancer cells *in vivo*. The components of the machinery controlling cortical actin assembly, however, in tumor cell migration have yet to be identified. We pursued the hypothesis that DIP-controlled mDia2 cytoskeletal remodeling plays a critical role in breast cancer cell movement through three-dimensional matrices. Using live-cell confocal imaging, we compared the effects of manipulating DIP and mDia2 activity in MDA-MB-231 (which typify protease-dependent, elongated/mesenchymal cell motility) and MDA-435S (representing protease-independent, rounded, bleb-associated ACM) breast cancer cell lines stably expressing fluorescent actin as they migrated through matrices. DIP LRR expression induced a rounded, amoeboid morphology in the normally elongated MDA-MB-231 cells and caused continuous nonapoptotic blebbing and invasion through the gel. Conversely, MDA-MD-435S cells, which normally have a more rounded morphology and whose invasion is dependent upon the contractile machinery (e.g., ROCK), reverted to a more elongated shape and were devoid of blebbing activity upon DIP depletion by shRNA expression. These results implicate DIP as a crucial pivot, via its regulation of actin nucleators, for promoting amoeboid-type, protease-independent mechanisms for cell migration and invasion. Understanding the role of DIP in controlling formin (de)activation and its effects on amoeboid cell movement may lend novel insight as to mechanisms controlling the amoeboid-based migration/invasion and metastasis of breast cancer cells and provide an alternate therapeutic avenue.

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P23-9: MITOTIC SPINDLE POSITIONING IN BREAST CANCER (CONCEPT STUDY)

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Significance: Estrogen receptor (ER) signaling plays a critical role in breast cancer development, and antiestrogen therapies are a mainstay of therapy. Understanding the effects of estrogen on microtubule-based processes such as spindle orientation may help explain how estrogens promote breast cancer development and guide the use of antiestrogen therapies. A three-dimensional system for assaying estrogen effects on spindle orientation will be useful for studying mechanisms and testing the activity of future antiestrogen therapies.

Background: In the uterus, estradiol treatment changed the orientation of mitotic spindles relative to the tissue plane, a major feature of epithelial polarity (Gunin, 2001). It is unknown whether spindle orientation is regulated in the breast. Estrogen-induced changes in spindle orientation could cause ductal hypertrophy and contribute to breast cancer development. Here we test two hypothesis: (1) breast tumors show abnormal mitotic spindle positioning and (2) ER signaling alters spindle positioning in cultured breast epithelial (MCF-10A) cells.

Methods:

Aim 1: Breast tissue will be obtained from women undergoing reduction mammoplasties for benign disease and cancer resections. Spindles will be imaged by microtubule immunofluorescence and DNA staining with high-resolution spinning disk confocal microscopy. Spindle angle will be compared in benign and malignant tissue, stratifying malignant tissue by ER status.

Aim 2: Recently, a group has engineered the benign breast cell line MCF-10A to express the ER and to undergo physiological ER signaling (Abukhdeir et al., 2006). We will determine spindle angle in these cells in the presence and absence of estradiol.

Preliminary Results: Collection of breast tissues is ongoing. In the interim, we have imaged spindles from mouse mammary tissues during pregnancy. We find the majority of spindles are oriented parallel to the apical cell surface (Figure 1). Spindles in MCF-10A cells expressing the estrogen receptor were randomly oriented in the presence and absence of estradiol. This suggests that spindle orientation in the breast depends on external factors not present in this culture system. Additional cell culture model systems are being pursued.

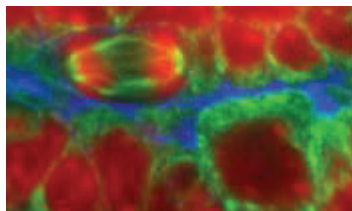


Figure 1. Spindles are oriented parallel to the apical cell surface in mouse mammary tissue.

Conclusions: Spindle orientation appears regulated *in vivo* but not in a cell culture model. During pregnancy, a state of high estrogen exposure, spindles were oriented parallel to the apical cell surface. This orientation would be expected to cause ductal expansion rather than branching.

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P23-10: ANTAGONISM OF TUBULIN AND ACTIN FILAMENT SYSTEMS REGULATES MICROTUBULE PROTRUSIONS IN HUMAN BREAST TUMOR CELLS

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Breast cancer remains the second leading killer of women in the U.S. today, usually due to metastatic spread of a primary tumor. Addressing this with novel therapeutic strategies necessitates a thorough understanding of the mechanisms of solid tumor progression. The cytoskeleton is a highly regulated network that governs many aspects of metastasis, including adherent cell motility, intracellular signal transduction and the invasive potential of transformed cells. The epithelial-mesenchymal transition (EMT) is characterized in part by the acquisition of dynamic cortical actin structure and is a hallmark of aggressive tumors. We have described highly dynamic tubulin-based protrusions of the plasma membrane, which are observed at high frequency in tumor cell lines of great metastatic potential in accordance with the classically invasive phenotype. Untransformed cells and tumor lines of low metastatic potential with rigid epithelial-like cortical actin structure demonstrate reduced frequency of protrusions, which have been termed microtentacles (mcTN). Indicative of their distinction from actin-based filopodia and invadopodia, depolymerization of actin enhances mcTN formation, as shown previously with Latrunculin-A (LA). Jasplakinolide (Jas), an F-actin binding agent, forces *de novo* polymerization and large-scale disruption of F-actin *in vivo*, increasing mcTN frequency in both nontumorigenic mammary epithelial cells and breast tumor cell lines. Known to impede cell growth at periods longer than 24 hours, the effects of Jas were demonstrated by 1 hour at 500 nM with no decrease in cell viability, even at 6 hours post treatment. Propidium iodide (PI) was excluded from cells with active mcTNs, thus Jas-enhanced protrusions are observed in viable, nonapoptotic cells and do not result from Jas-induced cytotoxicity. Immunofluorescent labeling revealed that Jas induces extensive polymerization and organization of α -tubulin into outwardly projecting filaments that extend from the cell periphery, concomitant with the collapse of F-actin. This effect was recapitulated for detubulated tubulin, a stable isoform that is known to localize along the length of individual mcTNs. Inclusion of the microtubule stabilizing agent, taxol, dramatically enhanced this effect in adherent cells and increased mcTN frequency in suspended cells. Cell aggregation assays demonstrated that cells treated with combinations of Jas and Taxol coalesced into large suspended colonies more rapidly than their untreated counterparts, indicating that mcTNs may affect cell-cell binding. These results describe a novel antagonism between actin and tubulin cytoskeletal networks in the context of membrane protrusions. Intravital microscopy studies implicate these novel tubulin mcTNs during the binding of circulating tumor cells to blood vessel walls during extravasation. Our current findings warrant caution with the use of taxol in the context of F-actin disruption. Since aggressively transformed cells often display disrupted F-actin, it remains possible that taxol could enhance the aggregation and extravasation of circulating tumor cells, which would promote metastasis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0423.

P23-11: LOSS OF THE POLARITY GENE SCRIBBLE COOPERATES WITH Myc DURING MAMMARY TUMORIGENESIS BY BLOCKING MYC-INDUCED APOPTOSIS

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Loss of function mutations in polarity genes, such as Scribble, induce neoplastic overproliferation of epithelial cells in *Drosophila*, suggesting that polarity genes function as tumor suppressors by regulating proliferation control. The role played by polarity genes, including Scribble, during tumorigenesis in mammals is poorly understood. Here, we demonstrate that downregulation of Scribble promotes Myc-induced transformation of mammary epithelial cells in culture and in mouse models of breast cancer by blocking Myc-induced cell death with no detectable effect on proliferation. Surprisingly, Scribble is required for Myc to activate the Rac-JNK (c-Jun N-terminal Kinase) pathway and trigger apoptosis. Inhibition of Rac or JNK activity phenocopied Scribble loss and blocks Myc-induced apoptosis indicating that the Scribble-Rac-JNK axis of signaling is a novel regulator of Myc-induced transformation of mammary epithelial cells. In addition, loss of Scribble blocks luminal apoptosis during acinar morphogenesis of breast epithelial cells in three-dimensional culture and promoted development of dysplastic mammary ductal outgrowth *in vivo*. So Scribble regulates cell death pathways during normal morphogenesis and Myc-induced trans-

formation of mammary epithelial cells. Thus, we demonstrate that mammalian Scribble genetically interacts with Myc and functions as a modulator of tumor growth by regulating cell death pathways.

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P23-12: VIMENTIN FILAMENTS SUPPORT EXTENSION OF TUBULIN-BASED MICROTENTACLES IN DETACHED BREAST TUMOR CELL LINES

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Solid tumor metastasis often involves detachment of epithelial carcinoma cells into the vasculature or lymphatics. However, most studies of cytoskeletal rearrangement in solid tumors focus on attached cells. We report for the first time that human breast tumor cells produce unique tubulin-based protrusions when detached from extracellular matrix. Tumor cell lines of high metastatic potential show significantly increased extension and frequency of microtubule protrusions, which we have termed tubulin microtentacles (mcTN). Our data indicates that microtentacles observed in detached cells are distinct from actin-based structures, such as invadopodia or podosomes, as they are not inhibited in the presence of actin-destabilizing agents such as Latrunculin-A or Cytochalasin-D. On the contrary, the microtentacles appear to become more flexible, motile, and in some instances, longer in presence of such agents. Our previous studies in nontumorigenic mammary epithelial cells showed that such detachment-induced microtubule protrusions are enriched in detyrosinated α -tubulin. However, amounts of detyrosinated tubulin were similar in breast tumor lines, despite varying microtentacle levels. Given that detyrosinated α -tubulin associates more strongly with intermediate filament proteins, we examined the contribution of cyokeratin and vimentin filaments to tumor cell microtentacles. Increased microtentacle frequency and extension correlated strongly with loss of cyokeratin expression and upregulation of vimentin, changes characteristic of the epithelial-to-mesenchymal transition associated with tumor progression. We also report the novel finding that vimentin-expressing, invasive breast carcinomas display a comparatively higher frequency of tubulin microtentacles following detachment than non-vimentin expressing, non-invasive cell lines. Microtentacle preservation following detachment indicates that vimentin coaligns with Glu-tubulin in microtentacles, while cyokeratin does not. Furthermore, time-lapse video shows that membrane microtentacles are rapidly disrupted by treatment with PP1/PP2A inhibitors that disassemble vimentin, decreasing microtentacle frequency thus demonstrating the dependence on vimentin for the integrity and persistence of such structures. Furthermore, disruption of vimentin reduced cell reattachment to extracellular matrix, suggesting a role that microtentacles have in cell adherence to both culture surfaces and extracellular matrix. Our results support a novel model in which coordination of vimentin and detyrosinated microtubules provides structural support for the extensive membrane protrusions observed in detached tumor cells and a possible advantageous mechanism for metastatic spread.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0383.

P23-13: THE EFFECTS OF LOCAL ANESTHETICS ON MICROTENTACLE PROTRUSIONS OF HUMAN EPITHELIAL AND BREAST TUMOR CELLS

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Detached breast tumor cells produce long, dynamic microtubule protrusions that actively promote reattachment of cells to each other and to surfaces. These microtubule protrusions have been termed tubulin microtentacles due to their mechanistic distinctions from both actin-based filopodia/invadopodia and tubulin-based cilia. Recent observations in mammary epithelial cells and breast tumor cells show that these microtentacles are enriched with vimentin intermediate filaments and depend on vimentin assembly for their extension. Within microtentacles, vimentin coaligns with a stable, detyrosinated form of alpha tubulin, in which the c-terminal tyrosine is removed, exposing a glutamic acid residue (Glu-tubulin). Given evidence that vimentin and Glu-tubulin are cross-linked by kinesin motor proteins, we tested the role of kinesins in microtentacle formation and function. The local anesthetics Lidocaine and Tetracaine have been shown to inhibit kinesins during *in vitro* motility assays as well as in single-molecule assays. Treatment of human nontumorigenic mammary epithelial cells and breast tumor cell lines with Lidocaine and Tetracaine led to a rapid centripetal collapse of vimentin filaments, as detected by immunofluorescence. Live-cell fluorescence video microscopy also demonstrated that Lidocaine and Tetracaine reduced the directed motility of intracellular vesicles. Both the collapse of vimentin and the cessation of vesicle motion indicate that cellular kinesin motor activity can be inhibited with Lidocaine and Tetracaine, which has not been shown previously. Measurements of cell viability with XTT or apoptotic induction with PARP cleavage did not show toxic effects at the concentrations of Lidocaine and Tetracaine that affected cellular kinesin activity. Treatment with Lidocaine or Tetra-

caine significantly inhibited the extension and motion of microtentacles in detached mammary epithelial and breast tumor cells. Video microscopy demonstrated a centripetal collapse of microtentacles after treatment with Lidocaine or Tetracaine that paralleled the time course of vimentin collapse. Lidocaine and Tetracaine also inhibited the homotypic aggregation of detached epithelial and breast tumor cells, a process that depends on tubulin microtentacles. In conclusion, the local anesthetics Lidocaine and Tetracaine can reduce cellular kinesin activity and inhibit the extension tubulin microtentacles and their ability to promote cellular aggregation. Our current data support a model in which the known ability of Lidocaine and Tetracaine to reduce the metastatic efficiency of circulating tumor cells could result from the inhibitory effects of these amine anesthetics on tubulin microtentacles.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0383.

P23-14: THE EXPRESSION OF THE MICROTUBULE-ASSOCIATED PROTEIN, Tau, IN DETACHED BREAST TUMOR CELL LINES ALTERS MICROTENTACLE FORMATION

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The sensitivity of breast cancer to chemotherapy is a primary concern for clinicians and patients. Though neoadjuvant and adjuvant treatment regimens greatly increase the rates of disease-free survival, there remains great variability in cancer sensitivities. To combat this, numerous pharmacogenomic studies have been undertaken to discover predictive molecular markers of breast cancer chemotherapy response. The goal of these studies includes the development of patient-specific treatment regimens with increased efficacy, shortened duration, decreased toxicity, and reduced costs. One marker to emerge from such a program is the microtubule-associated protein (MAP), tau. Tau is a classically neuronal MAP that binds to, stabilizes, and promotes polymerization of microtubules. In gene expression profiles of paclitaxel sensitive and resistant breast cancers, tau was differentially expressed. Tau mRNA expression directly correlated with paclitaxel sensitivity and inversely correlated with patient outcome. Specifically, patients who experienced pathologic complete response from chemotherapy that included paclitaxel had sensitive tumors with low tau mRNA expression. In contrast, patients who experienced residual disease or distant recurrence with paclitaxel-containing chemotherapy possessed resistant tumors with high tau mRNA expression. The mechanism for this response has been attributed to the binding competition of tau and paclitaxel for the same site on the microtubule polymer. To our knowledge, little is known of how tau expression impacts cancer invasiveness and metastasis. Our research focuses on unique tubulin microtentacles that form in detached breast tumor cells and that are implicated in the binding of circulating tumor cells to blood vessel walls during extravasation. We have demonstrated that microtentacles, enriched in detyrosinated tubulin and vimentin, facilitate the reattachment of suspended cells to other cells and to an extracellular matrix. This trait is enhanced in cells with a compromised actin cortex. We examined breast tumor cell lines of varying metastatic potential and found that those endogenously expressing high levels of tau showed extensive microtentacle formation upon suspension and actin disruption. To investigate the role of tau in microtentacle formation, tau was exogenously expressed in cell lines with little or without tau expression. In a cell line with low microtentacle frequency, tau expression significantly increased microtentacle formation upon actin disruption and suspension. Conversely, in a cell line with a natively weak actin cortex and high microtentacle frequency, exogenous tau expression significantly suppressed microtentacle formation. Our current model supports a biphasic role for tau in microtentacle formation. This indicates a potential mechanism by which tau expression can influence metastasis of breast tumor cells.

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P23-15: DECONSTRUCTING DIRECTIONAL CELL MOTILITY WITH SUBSTRATUM MICROPATTERNING

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The purpose of this project is to uncover the molecular mechanism controlling directed cell motility so as to develop novel antimetastasis therapeutics. We focus on the regulation of persistent cell movement by the dynamic activity of microtubule (MT) plus-ends. Our hypothesis is that molecular complexes at MT plus-ends coordinate cell polarization and motility by regulating the lifetime of focal adhesions. We have developed the Anisotropic Solid Microetching (ASoMic) technique for analysis of intracellular motility in cells of geometrically defined shapes. ASoMic is generally compatible with cell biological imaging modalities and enables quantitative analysis of cell biological processes (Kandere-Grzybowski et al., *Nat Methods* 10, 739, 2005.). First, we have developed a straightforward ASoMic-based cell polarization assay; here, synchronized molecular polarization of cell populations is observed on circular or tear-drop micro-patterned islands. Second, we address an element of our hypothe-

sis that proposes to test whether MT growth toward the adhesions is a random search or guided process. By quantitative analysis of directionality of all MT growth trajectories, we show that MT growth in both internal and peripheral cytoplasm is guided toward focal adhesions at the vertices of triangular cells (Kandere-Grzybowski et al., *Soft Matter* 3, 672, 2007). Finally, we have defined the determinants of MT-dependent, persistent single-cell movement. Taken together, these results may help to understand role of MT cytoskeleton in invasive behavior of breast cancer cells.

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P23-16: ELUCIDATING THE ROLE OF Hec1 IN MAINTAINING THE INTEGRITY OF CENTROSOMES AND MITOTIC SPINDLE TO PREVENT GENETIC INSTABILITY

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Tumorigenesis involves the accumulation of multiple genetic lesions that transform a normal cell into a malignant cell over time. Cytogenetic analysis of cancer reveals that a majority of these malignant cells contain gross chromosomal rearrangements, loss of genetic material, or gain of additional genetic material, all of which contribute to the process of tumorigenesis. These characteristics exemplifying genetic instability can be seen in cells deficient of one of the classical tumor suppressors: retinoblastoma (Rb), p53, and APC. The identification of Hec1 (highly expressed in cancer 1) using Rb as a bait in a yeast two-hybrid system has identified a novel and indispensable coil-coiled protein that interacts with a constellation of proteins important in facilitating proper chromosome segregation thereby maintaining the integrity of the genome. Hec1 has been shown to be highly expressed in rapidly dividing cells and breast cancer specimens, and this overexpression of Hec1 has served as a viable cancer target in two separate adenoviral RNAi therapies in mouse models. In yeast, faithful chromosome segregation requires the phosphorylation of Hec1 by Nek2. Human Hec1 can rescue scHec1 null yeast, but not scHec1 or hHec1 mutants that abrogate Nek2 phosphorylation at Ser 201 and Ser 165, respectively. We aim to determine the molecular mechanism and significance of this phosphorylation event in the human system.

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P23-17: MCF-10A BENIGN MAMMARY EPITHELIAL CELLS ENHANCE THE MALIGNANT PHENOTYPE OF MDA-231 BREAST CANCER CELLS IN VITRO AND IN VIVO

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Background and Objectives: Interactions between cancer cells and their microenvironment play a critical role in tumor development. It has been shown that stromal cells can enhance the tumorigenic growth of malignant or premalignant cells. However, normal epithelial cells are present in the environment of arising cancer as well. The purpose of this project is to investigate the interactions between human normal mammary epithelial cells and breast cancer cells.

Methods: As a model of breast cancer cells, we used MDA-231-T1AS, a subline selected for high tumorigenicity in mice. The MCF-10A cell line served as a model of normal mammary epithelial cells. MDA-231 cells and MCF-10A cells were transduced with red and green fluorescent proteins, respectively, to serve as lineage markers. We examined the interaction between the two cell lines in vitro using 3D matrigel assay and soft agar colony formation assay and in vivo using the nude mouse xenograft assay.

Results: In 3D matrigel co-culture, under conditions that allowed cells to aggregate, the malignant, invasive phenotype of MDA-231 cells was suppressed while the benign phenotype of MCF-10A cells was barely changed. In the soft agar assay, the cells (mixed, normal-only, and cancer only) were cultured in medium with low glucose and low serum content. After 21 days, the number of colonies was scored. Preliminary data indicated that the number of colonies formed in the mixed culture was increased 2–5-fold in comparison to cancer only. Using fluorescent microscopy, we determined that the colonies in the mixed culture were composed exclusively of cancer cells. In the nude mouse tumorigenicity assay, we mixed 1×10^6 of MDA-231 cells with 4×10^6 of MCF-10A cells and injected them into mammary fat pads of nude mice (mixed

tumors). As controls, we injected 1×10^6 of MDA-231 cells (cancer only), and 4×10^6 of MCF-10A cells (normal only). We determined that MCF-10A cells significantly accelerated the growth of tumors formed by MDA-231 breast cancer cells although the normal cells did not contribute to the tumor mass. Proliferation rates assessed in tumors harvested at days 7 and 14 post-inoculation were not different between mixed and cancer-only groups. Analysis of histological sections of 7-day tumors showed that mixed tumors contained a fluid and debris-filled cyst surrounded by a layer composed of MDA-231 cells, MCF-10A cells, and infiltrating inflammatory cells. By day 14, almost all of the MCF-10A cells disappeared and the tumors developed a solid structure. To determine whether the normal cells actively secrete factors that enhance the tumorigenic growth of cancer cells, we have mixed cancer cells with paraformaldehyde-fixed normal cells. We observed that the growth of the tumors was equally enhanced by fixed cells.

Conclusions: In matrigel co-culture, MCF-10A cells imposed the benign phenotype on MDA-231 cells. The results of the soft agar assay indicated that MCF-10A cells provided factors that enhanced the survival and proliferation of MDA-231 cells under low glucose and low serum conditions. In the nude mouse assay, MCF-10A cells dramatically enhanced the growth of MDA-231 breast cancer cells. Similar effect was elicited by paraformaldehyde-fixed MCF-10A cells, suggesting that in this assay the normal cells need not be alive to enhance the tumorigenic growth of breast cancer cells.

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P23-18: DEFECTIVE MAMMARY GLAND DEVELOPMENT BY NHERF1 GENE DELETION

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NHERF1 is a putative tumor suppressor gene in mammary gland. We reported that loss of heterozygosity (LOH) at the *NHERF1* locus is a frequent event in human breast carcinoma (~60%), among which intragenic mutations of *NHERF1* are identified in a subset of cases. The *NHERF1* LOH is strongly associated with decreased gene expression. *NHERF1* interacts with a variety of proteins through its tandem PDZ domains to regulate a number of signal transduction pathways. We have shown that *NHERF1*, through its tandem PDZ domains, interacts with the carboxyl tail of PTEN (PDZ-binding motif). This interaction accelerates the turnover of PI3K-Akt activity that is initiated by upstream growth factors, indicating that *NHERF1* plays a key role in suppressing cell survival pathway. In the present study, we used *NHERF1* knockout (KO) mice to determine the effect of *NHERF1* on mammary gland development and tumorigenesis. In *NHERF1* KO mice, we found that deletion of 2 *NHERF1* alleles resulted in alveolar hyperplasia in mouse mammary gland. Interestingly, hemizygous deletion led to a similar hyperplastic phenotype as that observed in the wild-type mice, albeit to less extent. The mammary hyperplasia observed in *NHERF1* KO mice was accompanied by increased proliferation as measured by BrdU incorporation, suggesting that one of the *NHERF1* functions is to suppress cell proliferation. During mammary involution, *NHERF1*(-/-) and (+/-) mice had fewer ductal epithelial cells that underwent apoptosis than (+/+) mice as detected by TUNEL labeling, suggesting that *NHERF1* is a pro-apoptotic factor. This mechanism may underlie the hyperplastic morphology found in *NHERF1* KO mice. We also measured the level of p-Akt and p-Erk in mammary tissues from *NHERF1* KO mice by immunoblotting; biallelic deletion of *NHERF1* gene led to markedly elevated p-Akt and p-Erk, while monoallelic deletion of *NHERF1* showed modest but consistent increase in p-Akt level. Using a human breast carcinoma tissue array, we found that *NHERF1* protein expression was decreased or lost in 63% of the neoplastic specimens. By contrast, all histologically normal mammary gland tissues examined had positive *NHERF1* expression. We found that *NHERF1* loss occurred as early as TNM-I stage (>50% of cases), which did not appear to become more frequent as disease progresses. Interestingly, loss of *NHERF1* expression was shown to be associated strongly with triple-negative phenotype, a subgroup of aggressive breast carcinoma that is related to resistance to therapies and poor prognosis. Together, our results indicate that *NHERF1* loss is a frequent event in human breast carcinoma that occurs early in the tumorigenesis. This loss leads to hyperplastic phenotype in mammary gland through increased proliferation and lowered apoptosis, which may result from elevated p-Akt and p-Erk. The similar phenotypic change found in heterozygous *NHERF1* KO mice implicates a haploinsufficient *NHERF1* activity in mammary gland. These pathobiologic effects resulted from *NHERF1* loss are expected to promote mammary neoplasia.

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FUNCTIONAL STUDY OF BIOLOGICAL MOLECULES I

Poster Session P24

P24-1: EXPRESSION OF THE ERYTHROPOIETIN RECEPTOR (EPOR) BY MAMMARY EPITHELIAL CELLS RESULTS IN A PREMALIGNANT PHENOTYPE

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Background/Objectives: In vitro studies have demonstrated the presence of functional erythropoietin receptors (EpoR) on a variety of tumor cells and cell lines. In addition, data from a growing number of clinical trials have suggested an effect of erythropoiesis stimulating agents (ESAs) such as erythropoietin (Epo) on the growth and survival of primary tumors. Data indicate that in the majority of cases the normal (i.e., nontumorigenic) counterparts of these tumor cells also express EpoR. The significance of extra-hematopoietic EpoR in general and their potential role(s) in the growth and survival of cancer cells in particular are not well understood. One notable exception to the appearance of EpoR on both normal and malignant cells is that of mammary epithelial cells. RT-PCR and immunohistochemical studies have demonstrated that normal mammary epithelial cells do not express EpoR (gene or protein) while breast cancer cells express functional EpoR. This differential expression of EpoR has led us to hypothesize that acquisition of EpoR by mammary epithelial cells may impact the oncogenic process.

Methodologies: To test the hypothesis that EpoR acquisition is involved with malignant transformation of mammary epithelial cells, we stably transfected nontumorigenic MCF10A mammary epithelial cells with a human EpoR cDNA in pcDNA3.1 to derive MCF10A/EpoR cells. As control, MCF10A cells were transfected with pcDNA3.1 vector alone to generate MCF10A/vector cells. RT-PCR was used to confirm the expression of EpoR in MCF10A/EpoR (but not MCF10A/vector) cells. Two- and three-dimensional cell culture, growth and survival assays, signal transduction assays, and telomerase assays were used to enumerate changes in in vitro biology of MCF10A/EpoR cells related to their EpoR acquisition.

Results: MCF10A/EpoR cells have altered morphology in culture, and their growth rate is increased significantly over that of MCF10A or MCF10A vector cells. MCF10A/EpoR cells lose their dependence on EGF and insulin, and they respond to Epo in culture. They become anchorage independent for growth, forming colonies in soft agar whose number/size is increased by Epo. While MCF10A and MCF10A/vector cells fail to express telomerase, MCF10A/EpoR cells have telomerase activity that is increased further by prior treatment of the cells with Epo. The paclitaxel sensitivity of MCF10A/EpoR cells is decreased in chronically Epo-treated cells. Interestingly, acquisition of EpoR expression by MCF10A/EpoR cells results in subsequent expression of the (endogenous) Epo gene in these cells.

Conclusions: Our data suggest that acquisition of EpoR by mammary epithelial cells is, at the very least, permissive for anchorage-independent, growth factor-independent growth of the cells in vitro. These are changes consistent with the development of a premalignant phenotype. Subsequent expression of endogenous Epo by the cells suggests that an autocrine and/or paracrine Epo-EpoR growth regulatory loop may drive their increased growth and survival. The data support our hypothesis that EpoR acquisition by mammary epithelial cells may be integral to malignant transformation and may contribute to the oncogenic process in breast cancer. The data also support the potential development of the EpoR—and endogenous Epo—as novel therapeutic targets in breast cancer.

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P24-2: Akt1, NOT Akt2 IS INVOLVED IN RhoC GTPASE-MEDIATED INVASION OF INFLAMMATORY BREAST CANCER CELLS

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Inflammatory breast cancer (IBC) is arguably the most deadly form of breast cancer. This is primarily due to the propensity of IBC to invade the dermal lymphatics of the breast and metastasize rapidly throughout the body leading to the rapid demise of the patient. Previously, we demonstrated that RhoC GTPase was overexpressed and active in IBC. Furthermore, we demonstrated that expression of RhoC was responsible for the majority of the invasive IBC phenotype. Treatment of IBC cells with farnesyl transferase inhibitor (FTI) led to inhibition of the RhoC-induced phenotype. However, RhoC is not a direct target for FTI action. RhoB GTPase has been shown to be a putative target of FTI action. Inhibition of farnesylated RhoB (RhoB) by FTI leads to an increase in geranylgeranylated RhoB (ggRhoB). Introduction of ggRhoB in IBC cells recapitulates the effects seen with FTI. RhoB regulates cellular localization of Akt1 affecting its activation. In endothelial cells, FTI treatment or ggRhoB expression leads to mislocalization and decreased activation of Akt1. We have demonstrated that RhoC contains a putative Akt phosphorylation site and we have evidence that RhoC is an Akt substrate phosphorylating the GTPase at serine71.

Pharmacologic inhibition or suppression of Akt1 expression by siRNA leads to a statistically significant decrease in the ability of the SUM149 cells to invade through Matrigel. Similarly, introduction of RhoC S71A mutant or a wild-type Pten into the IBC cells leads to a significant decrease in the cells invasive capabilities. In contrast, inhibition of Akt, introduction of the RhoC S71A mutant or wild-type Pten did not affect the invasive ability of the MDA231 or MDA435 cell lines. Although the invasive capabilities of the SUM149 IBC cell line was affected when Akt1 was inhibited, the survival and levels of apoptosis were not. Conversely, inhibition of Akt1 in the MDA231 and MDA435 cells led to increased cell death.

Evidence from other laboratories has suggested that Akt2 is involved in breast cancer cell migration. Our results suggest that Akt2, although expressed, does not play a significant role in SUM149 IBC cell invasion or cell survival. However, we found that Akt2 does have a significant role in the invasive capabilities of the non-IBC MDA231 and MDA435 cell lines. Taken together these results suggest another significant difference between inflammatory and non-inflammatory breast cancers and can lead to the development of novel therapies to inhibit the metastatic spread of IBC.

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P24-3: THE ROLE OF CHEMOKINES IN CANCER AND THE DEVELOPMENT OF RECEPTOR ANTAGONISTS

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Background, Objectives, and Impact: In parallel with their role in controlling cell migration during immune responses, chemokines/receptors have been implicated in metastasis. There is also emerging evidence that chemokines may contribute to survival and proliferation of metastasized cells as well. Breast cancers frequently show high expression of the receptors CXCR4 and CXCR7, while they are not expressed in normal breast epithelia, and they preferentially metastasize to tissues where the ligand, CXCL12, is constitutively expressed. The first goal of the proposed study is to characterize the functional roles of CXCR4 and CXCR7 in breast cancer with the hypothesis that CXCR4 and CXCR7 confer growth and survival advantages. If this is the case, inclusion of inhibitors of CXCR4 and CXCR7 in chemotherapy could improve survival, not only by inhibiting cell migration, but by interfering with the ability of metastasized cells to survive in environments that would be otherwise inhospitable. The second objective is to evolve ligand derived antagonists of the above receptors using a combination of rational design and phage display. Our goal is to produce chemokine variants that bind their receptors with high affinity but do not induce receptor signaling.

Methods and Results: *Objective 1:* Although it is well established that CXCR4 contributes to metastasis, we hypothesize that it also confers growth and survival advantages to the cells. We have shown that MDA-MB231 cells previously selected from metastasized lesions express high levels of CXCR4 compared to the same cells from ATCC. These cells produce tumors more rapidly than the ATCC cells in SCID mice, suggesting a potential role of CXCR4 in cell growth. The ATCC cells do eventually grow, and when the tumors are isolated they have elevated expression of CXCR4 compared to the initial cell population suggesting either upregulation of CXCR4, or that the cells that grow aggressively are the small population of CXCR4 cells in the initial population. To address this question, we are sorting the ATCC cells to enrich for high CXCR4 expressors. The in vivo metastasis model will be used to determine whether these cells mimic the growth and metastatic behavior of the cells previously selected from the metastasized lesions. In contrast, CXCR4 does not appear to confer a growth advantage to cancer cells in vitro. However, based on preliminary microarray data, numerous receptors related to growth are upregulated in the metastasized cells upon CXCL12 stimulation suggesting an indirect effect. Experiments to further investigate this finding are under way. Similar studies are being conducted with CXCR7. Although CXCR7 does not induce migration, it is clear from our preliminary data that high-CXCR7 breast cancer cell populations have enhanced rates of proliferation over unsorted populations in vitro.

Objective 2: To engineer chemokine antagonists, we have created libraries of modified CXCL12 displayed on phage. The library design was based on our knowledge of the molecular details of chemokine-receptor recognition and signaling, beginning with scaffolds that are biased toward receptor antagonism. Next we will select tight binding variants by panning against receptor bearing cells and characterize their ability to block receptor signaling. The development of antagonists for chemokine receptors will enable us to probe the therapeutic value of such inhibitors in breast cancer treatment.

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P24-4: MECHANISM OF EGF NEUTRALIZATION BY ARGOS-LIKE MOLECULES

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Members of the Epidermal Growth Factor (EGF) Receptor family (also known as HER/ErbB family receptors) and their activating ligands are essential regulators of diverse developmental processes, and their inappropriate activation is a key feature of many human cancers. In particular, HER2/ErbB2 is well known to be associated with a significant proportion of breast cancers and is the target of the therapeutic Herceptin antibody. Tumor initiation and progression can result from overexpression of receptors or their activation by mutation. In these cases, agents that target and inhibit the receptors directly can be of therapeutic value. Both antibody-based and small molecule inhibitors of EGFR and HER2/ErbB2 have proven valuable in breast and other cancers. Alternatively, receptors from the ErbB family may signal aberrantly (and promote cancers) if their activating ligands are produced at inappropriately high or uncontrolled levels. In these cases, agents that can inactivate or neutralize HER/ErbB receptor-activating growth factors offer another avenue for therapeutic intervention. Our aim is to develop antagonists of HER/ErbB receptor-activating growth factors for use in breast cancer treatment. Our approach is to exploit lessons we have learned from studies of a protein called Argos, which is a secreted, long-range antagonist of EGF receptor signaling in *Drosophila melanogaster*. Argos was first identified in 1994, and was proposed to function as a direct inhibitor of the *Drosophila* EGF receptor. In 2004, we showed that Argos actually functions by binding to and sequestering growth factor ligands. Argos binds directly to the ligand's EGF domain and does not interact with the receptor. We have shown that Argos binds strongly to all four EGF-like ligands produced in *Drosophila*. It also binds (albeit weakly) to human EGF. Our aims are to identify functional orthologs of Argos in humans and to develop Argos analogs that will neutralize human EGF-like ligands and may ultimately be of therapeutic value. We recently determined a high resolution crystal structure of Argos bound to an EGF receptor ligand, the details of which will be described at the meeting. Argos has a unique structure and lacks an EGF domain—by contrast with initial suggestions. Its mechanism of ligand neutralization will also be described, as will the prospects for using this information to develop novel anti-EGF therapeutics. An undiscovered mammalian Argos counterpart could exist among previously unidentified structural homologues of Argos. The studies that will be described illustrate requirements for artificial EGF-trapping proteins that could be valuable anti-cancer therapeutics.

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P24-5: HuR BINDS TO A NOVEL 99 nt NON AU-RICH ELEMENT IN THE 3 PRIME UNTRANSLATED REGION OF *c-fms* RNA, REGULATES C-FMS PROTO-ONCOGENE EXPRESSION, AND IS AN INDEPENDENT POOR PROGNOSTIC FACTOR IN BREAST CANCER

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Background: The role of RNA binding proteins in cancer biology is increasingly being recognized. The nucleocytoplasmic shuttling and AU-rich RNA binding protein HuR stabilizes several target mRNAs, encoding cytokines and oncogenes. Stabilization of mRNAs can occur in both nuclear and cytoplasmic cell compartments. Expression of the proto-oncogene *c-fms*, whose 3' untranslated region (3'UTR) is not AU-rich, can be regulated on the basis of mRNA stabilization and its expression is associated with metastasis and poor prognosis in breast cancer patients. Physiologic levels of glucocorticoids (GC) markedly up-regulate *c-fms* expression.

Materials and Methods: RNA gel shift assays, and UV crosslinking label transfer assays were used to demonstrate specificity of binding of HuR protein to 3' UTR *c-fms* RNA. In vivo binding of HuR to *c-fms* RNA was demonstrated by immunoprecipitation-RT-PCR assays. Silencing RNA targeted to HuR was used to demonstrate regulation of *c-fms* mRNA and protein by HuR. A large cohort tissue microarray of 670 human breast cancer specimens was used to investigate the clinical role of both nuclear and cytoplasmic HuR expression, since HuR shuttles between the nucleus and the cytoplasm.

Results: We define a novel 99nt non AU-rich containing region in the 3' UTR of *c-fms* RNA as a target binding motif for HuR. Deletion of the mid 69nt of the target motif abolishes binding in UV crosslinking assays of recombinant HuR protein with *c-fms* riboprobes containing the deletion mutant compared to the wildtype *c-fms* riboprobe. In vivo assays of HuR/*c-fms* RNA protein binding in BT20 breast cancer cells after RNA protein crosslinking and immunoprecipitation for HuR in the lysates, with RT-PCR performed for *c-fms* RNA, demonstrated in vivo binding of HuR to 3'UTR *c-fms* RNA. Northwestern analyses showed that the 99nt *c-fms* RNA element binds several proteins, including HuR, in BT20 cells. Silencing of HuR significantly inter-

feres with GC stimulation of *c-fms* mRNA and protein. By tissue microarray, we find that increased nuclear HuR expression was significantly associated with lymph node metastasis ($p=0.0371$) and independently with poor survival ($p=0.0319$, relative risk 1.45). Breast tumors with nuclear HuR, co-expressed *c-fms* in the cytoplasm ($p=0.0007$).

Conclusions: We are the first to describe that HuR specifically bound to a 99nt non AU-rich element in 3'UTR *c-fms* RNA, and regulated its expression. This 99nt element likely represents a target for protein binding. Furthermore, in the largest breast cancer cohort studied to date, we found that nuclear, but not cytoplasmic, HuR expression was an independent poor prognostic factor. Human breast cancer cells in vivo are continuously exposed to circulating GCs and we find GC stimulation of *c-fms* to be largely dependent on HuR's presence. In line with this, we observe that HuR was co-expressed with *c-fms* in the breast tumors. Collectively, our findings suggest that HuR may play a supportive role for *c-fms* in breast cancer progression.

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P24-6: ABSTRACT WITHDRAWN

P24-7: THE ROLE OF AN ONCOGENIC PROTEIN PHOSPHATASE IN A VIRUS PROTEIN-MEDIATED CHEMOSENSITIZATION

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Overexpression of a wild-type p53-inducible serine-threonine phosphatase Wip1, through gene amplification has recently been found in a variety of human cancers, including neuroblastomas (28%), breast (16%) and ovarian (25%) cancers. The Wip1 gene is located in the 17q23 amplicon, a novel region of amplification that has been linked with a poor prognosis of breast and ovarian cancer patients. Although expression of Wip1 is induced by p53 following irradiation, it negatively regulates p53 functional activation. In addition, Wip1 also negatively regulates cell cycle checkpoints via dephosphorylation of p53 protein, p38, Chk1, and Chk2 kinases. It is very likely that overexpression of Wip1 in human tumors may confer resistance to DNA damaging drugs (such as cisplatin, adriamycin, VP-16, etc.) and microtubular inhibitors, such as paclitaxel. Earlier reports indicate that up-regulation of phosphorylated p38 by adenoviral early 1a (E1A) protein contributed to E1A-mediated sensitization to anti-cancer drug-induced apoptosis (Liao Y, et al., MCB, 2003). To further investigate if Wip1, an upstream protein phosphatase of p38, also plays a role in E1A-mediated chemosensitization, we measured expression levels of Wip1 protein in both parental and E1A-expressing breast cancer cell line with known Wip1 amplification, such as MCF-7 and MCF-7/E1A. The expression levels of Wip1 proteins were dramatically reduced in the E1A stable clones in comparison with that of the parental and vector-transfected stable cells. In addition, when knocking down Wip1 expression by specific short hairpin (sh) RNA against Wip1 conferred sensitivity MCF-7 cells to anti-cancer drugs. Whereas, when re-introducing Wip1 in E1A-expressing cells, the expression of Wip1 abrogated E1A-mediated sensitization to anti-cancer drugs. Similar results were also observed in Wip1 (-/-) mouse embryo fibroblasts (MEFs). When exposed to anti-cancer drugs, the Wip1 (-/-) MEF cells were more sensitive to anti-cancer drug-induced apoptosis than the Wip1 (-/+) MEF cells. While re-introducing Wip1 expressing by introducing Wip1 cDNA plasmid into the Wip1 (-/-) MEFs confers resistance to anti-cancer drug-induced apoptosis. In addition, the drug sensitivity and percentage of apoptotic cells correlated with the relative expression levels of phospho-p38, p53, and Wip1 protein in the presence or absence of anti-cancer drugs. Our data suggest that targeting Wip1 expression is a novel approach for sensitization of Wip1 overexpressing cancer cells to anti-cancer drugs.

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P24-8: MODELING THE NUCLEOCYTOPLASMIC SHUTTLING OF THE ErbB-4 ICD

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ErbB-4 is a receptor tyrosine kinase that undergoes regulated intramembrane proteolysis to release its ICD intracellular domain into the cytoplasm. The ICD is present in the cytoplasm and/or nucleus of cells in both normal and cancerous tissues. In the cytoplasm, the ICD interacts with multiple proteins and regulates apoptosis through a mitochondria-dependent mechanism. The ICD also interacts with transcription factors in the cytoplasm and acts as a shuttling factor to transport these factors into the nucleus where they regulate genes important for differentiation. Thus, the localization of the ICD between the cytoplasm and nucleus is a key determinant to whether ICD induces apoptosis or differentiation. To understand the regulatory steps that guide ICD subcellular localization, we determined the levels of ectopically expressed ICD in the cytoplasm and nucleus in normal mammary epithelial cells. Here we show that mutation of either the dimerization domain or a putative PKC phosphorylation site re-

duces the amount of nuclear ICD. Using real-time, quantitative microscopy, we demonstrate that these mutants have a decreased rate of nuclear import. In addition, these ICD mutants have a reduced mobility in the cytoplasm compared to wild-type ICD.

In the nucleus but not the cytoplasm, wild-type ICD has a reduced diffusion rate that is consistent with it being part of a high molecular weight complex. The presence of this complex was further supported by size exclusion chromatography experiments showing that the ICD is part of high molecular weight complex.

Together, these data show that the subcellular localization of the ICD is mainly regulated at the level of nuclear import and that in the nucleus, but not the cytoplasm, the ICD is part of a high molecular weight complex.

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P24-9: ROLE OF THE DEUBIQUITINATING ENZYME UCH37 IN TUMORIGENESIS

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The ubiquitin-proteasome system (UPS) regulates diverse functions, including protein degradation, cell cycle progression, antigen presentation, signal transduction, transcription, DNA repair, endocytosis, and protein trafficking. The covalent modification of proteins with Ub alters protein localization, activity, and/or stability. A sequence of enzymatic reactions catalyzed by Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3), transfer Ub to the target protein. Ub conjugation is reversed by the action of deubiquitinating enzymes (DUBs), a group of primarily cysteine proteases that cleave Ub from substrate proteins.

Studies on the Ub-proteasome system have identified several biochemical functions for DUBs in the editing and processing of Ub chains on substrate proteins and in the recycling of Ub monomers. However, relatively little is known about the substrates and specificity of the approximately 90 DUBs encoded in the human genome or the pathways in which they act. Several DUBs have been linked to cancer-related processes, including CYLD, a tumor suppressor protein that regulates NF-κB signaling; USP2a and HAUSP, which regulate the p53/Mdm2 pathway; and USP28, which regulates stability of the myc transcription factor.

We are interested in the role of the deubiquitinating enzyme UCH37 in tumorigenesis. UCH37 is one of three proteasome-associated DUBs that remove ubiquitin residues from proteins prior to their proteasomal degradation. We have found that shRNA-based knockdown of UCH37 protein in the human breast cancer cell line MDA-MB-231 reduces colony formation in soft agar and tumor growth in vivo but does not affect anchorage-dependent proliferation in vitro. We are undertaking a biochemical approach to investigate a mechanism for this observation using a modified tandem affinity purification of UCH37 and interacting proteins. We present a novel function for the DUB UCH37 in tumorigenesis in the MDA-MB-231 breast cancer cell line.

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P24-10: A NOVEL NONCODING RNA REGULATES MAMMARY EPITHELIAL CELL PROLIFERATION AND SURVIVAL

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Transcriptome analysis of several model organisms has revealed an unexpectedly large number of transcripts that do not contain open reading frames and are predicted to function as regulatory noncoding RNAs (ncRNA). As the appreciation for the importance of ncRNAs has just recently emerged, only a small percentage of these transcripts have been assigned a function; however, it is becoming increasingly clear that ncRNAs may play critical roles in gene regulatory processes during development and differentiation. mPINC (mouse pregnancy-induced noncoding RNA) is a large, polyadenylated ncRNA, which is alternatively spliced to give rise to a 1.0 kb form (mPINC 1.0) and a 1.6 kb form (mPINC 1.6). Expression of mPINC is upregulated in mammary epithelial cells (MECs) during pregnancy, and its expression persists in the epithelial cells that remain in the mammary gland after involution. In vitro studies of mPINC in HC11 cells, a mammary epithelial cell line derived from a mid-pregnant mouse, show that the expression and localization of the splice forms are differentially regulated throughout cell cycle progression. Knockdown of each splice form in HC11 cells suggest that mPINC 1.0 functions in regulating cell survival whereas mPINC 1.6 may play a role in modulating cell cycle progression. Our preliminary in vivo data suggest that mPINC 1.0 is essential for normal lobuloalveolar development during

pregnancy. In addition, further splice forms of mPINC 1.0 and 1.6 have been identified, which also appear to be differentially regulated during postpubertal development. Our hypothesis is that the splice forms of mPINC play an essential role in regulating survival and cell cycle progression of distinct populations of epithelial cells during the cyclical development of the postpubertal mammary gland. Recently, we performed qRT-PCR of mPINC splice forms during mammary gland development. Interestingly, mPINC 1.0 is expressed most abundantly at day two of involution when many alveolar cells are beginning to undergo programmed cell death, perhaps functioning as a survival factor in a distinct population of MECs, which may become the milk-producing alveolar cells in future pregnancies. We are further investigating the in vivo function of the splice forms of mPINC using lentiviral vectors that express short hairpin RNAs (shRNAs) to knockdown mPINC 1.0 and 1.6 in mammary gland transplants. A conditional knockout of the entire mPINC locus is also being generated to assess the effect of loss of all of the splice forms in the mammary gland. Finally, to further elucidate the function of this ncRNA we plan to identify proteins that bind mPINC transcripts and confirm binding in vivo.

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P24-11: THE ROLE OF LIMK1 SUBCELLULAR LOCALIZATION IN BREAST CANCER MIGRATION AND INVASION

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Background and Objectives: One of the main areas of investigation in the Gutierrez-Hartmann lab is the study of tumorigenesis and metastasis in breast cancer. Recently, LIM-kinase 1 (LIMK1) has become a key focus of investigation for its role in promoting metastatic behavior in models of human cancer. Increased expression of the serine/threonine kinase, LIM kinase 1 (LIMK1), has been shown to mediate invasion of tumor cells in both in vitro in matrigel invasion assays, as well as in vivo in mouse and rat models of breast cancer metastasis. Western blots from breast cancer patient biopsies have revealed increased expression of LIMK1. Important to my work, LIMK1 has been shown to shuttle between the nuclear and cytoplasmic compartments of the cell, and further, has been shown to directly phosphorylate and activate nuclear cyclic AMP response element binding protein (CREB) during neuronal differentiation. I propose that the increase of invasiveness associated with increased LIMK1 expression can be directly attributed to an enhanced nuclear localization of LIMK1.

Methods: I have chosen to use two distinct breast cancer cell lines, MCF-7 and MDA-MB-231. MCF-7 cells are estrogen dependent for growth and are typically not invasive in vitro, whereas MDA-MB-231 cells display strongly invasive properties. Thus, these cells will not only allow me to test gain and loss of function with regard to nuclear LIMK1 expression, but also to ensure that the observed effects are not cell-type specific. I have tagged recombinant forms of LIMK1 with a green fluorescent protein (GFP) tag to allow me to track the subcellular location of the GFP-fusion protein in real time via fluorescent microscopic imaging. These GFP-LIMK1 fusions were inserted into lentiviral vectors and transduced into MCF-7 and MDA-MB-231 cells, with stable populations selected with G418. I have generated MDA-MB-231 cells expressing wild-type [WT GFP-LIMK1] and a site-specific mutation of the nuclear export sequences (NES) of LIMK1 [GFP-LIMK1(A5)].

Results: The WT GFP-LIMK1 is expressed throughout the cell, whereas GFP-LIMK1(A5) is enriched in the nucleus. Stable MCF-7 cells expressing these same LIMK1 constructs are currently being expanded and characterized. The results of transwell filter invasion assays reveal that the WT GFP-LIMK1-expressing cells are more invasive than GFP-only control MDA-MB-231 cells, as has been previously reported. Notably, the GFP-LIMK1(A5)-expressing cells are substantially more invasive than the GFP-only control and the WT GFP-LIMK1-expressing cells.

Conclusions: These data show that increased LIMK1 expression results in increased cell invasion and that nuclear LIMK1 enrichment mediates even greater invasiveness, providing preliminary support of my hypothesis. Particularly important, however, is that I have established an invasion assay that identifies a functional property for the nuclear enrichment of LIMK1. Using this assay, I now plan to generate selected site-specific mutants of LIMK1, for example, mutating the NLS alone or mutating the kinase domain in the A5 background, to determine if cytoplasmic enrichment or nuclear kinase activity, respectively, contribute to the increased LIMK1-mediated motility. Similarly, mutations of domains that may be involved with the putative co-activator function of LIMK1 (e.g., LIM domains) will be targeted and mutants analyzed.

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P24-12: EVALUATING THE ROLE FOR FoxA1 IN MAINTAINING THE LUMINAL PHENOTYPE OF THE NORMAL MAMMARY GLAND AND BREAST CANCER CELLS

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Breast cancer is not a single disease but consists of at least five distinct subtypes: luminal A, luminal B, Her2+, basal-like, and normal-like. These subtypes can be discriminated with hierarchical clustering of human breast tumor cDNA microarray data and may be used to predict prognosis. Not surprisingly, known mediators of mammary cell survival and proliferation, such as the Estrogen Receptor- α (ER α), drive breast tumor clustering. ER α expression is significantly associated with less aggressive cancers defined as luminal-type, while loss of ER α is associated with more aggressive cancers defined as basal-type. While ER α is useful for predicting breast cancer subtype and prognosis, there are tumors that lack ER α , yet still cluster with the luminal subtype. This suggests that although ER α expression facilitates luminal classification, it is not necessary, and indicates that there are additional genetic factors that dictate a breast cancer luminal phenotype. One potential master regulator is the forkhead box transcription factor FoxA1. FoxA1 is a transcription licensing factor and is necessary for the expression of several ER α target genes. In addition, FoxA1 expression correlates with ER α -positivity, the luminal subtype, and patient survival. To further elucidate the role of FoxA1 in breast carcinogenesis, we immunohistochemically screened a cohort of human breast cancer tumor sections. As anticipated, we detected strong FoxA1 protein expression (intensity score ≥ 2) in the majority of ER α -positive tumors (96.9%). More importantly, we also detected strong expression (intensity score ≥ 2) in a subset of the ER α -negative human breast tumors (46.2%). Consistent with the human tumor data, FoxA1 expression is also elevated in luminal breast cancer cell lines regardless of ER α status. These data lead to the hypothesis that FoxA1 operates independently of ER α to maintain the luminal phenotype and is necessary for perpetuating the reduced aggressiveness of luminal breast cancers. Several processes of mammary gland development such as invasion, proliferation, resistance to apoptosis, and angiogenic remodeling parallel tumorigenesis, thus, we also assessed the role that FoxA1 may play in mammary gland development. Pronounced FoxA1 expression occurs in the body cells (i.e., luminal progenitor cells) of normal mouse mammary terminal end buds (TEBs), suggesting FoxA1 may be required for proper generation and maintenance of mammary luminal progenitor cells. Consistent with this hypothesis, the number of FoxA1 immunoreactive cells progressively declines with pregnancy and lactation. Moreover, we found that there is a decrease in epithelial cell proliferation, ductal length, and ductal branching in the mammary glands of pubertal FoxA1 heterozygous null mice compared to wild-type animals. In summary, when compared to ER α expression, FoxA1 more strongly associates with the breast cancer luminal phenotype. FoxA1 also displays haploinsufficiency, with loss of one allele leading to a disruption of proper mammary gland ductal morphogenesis, most likely due to a decrease in epithelial luminal lineage precursors.

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P24-13: MULTIPLE MECHANISMS FOR THE REGULATION OF MYOSIN-IIA ASSEMBLY: S100A4 BINDING AND HEAVY CHAIN PHOSPHORYLATION

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Two regulatory mechanisms modulate myosin-IIA filament disassembly: (1) binding of S100A4 to the C-terminal end of the coiled coil and (2) heavy chain phosphorylation on the tailpiece. In vitro, S100A4 regulates non-muscle myosin-IIA assembly and promotes the monomeric, unassembled state of myosin-IIA. In vivo studies show that S100A4 enhances cell polarization and motility. We propose two possible models by which S100A4 can elicit these in vivo effects: (1) the sequestration of myosin-IIA monomers and (2) the enhancement of the dynamics of myosin-IIA filament turnover. To investigate these two models of S100A4 mediated regulation of myosin-IIA assembly we are using quantitative photobleaching methods on EGF-stimulated MDA-MB-231 cells expressing fluorescently-tagged S100A4 or myosin-IIA. FRAP analysis on EGF stimulated GFP-NMHC-IIA cells shows that the half-time of recovery in the cell edge is 3-fold slower than the half-time of recovery in the perinuclear region. S100A4-depletion results in increased filament turnover in the edge region, whereas recovery in the perinuclear region is not affected. Preliminary FRAP experiments on cells expressing GFP-S100A4, indicate that S100A4 is highly mobile in the edge region. In addition to S100A4 binding, recent studies demonstrate that phosphorylation on S1943 of the myosin-IIA heavy chain enhances lamellipod extension and cell migration. To evaluate the contribution of heavy chain phosphorylation to filament turnover in vivo, we are examining assembly/disassembly dynamics in cells expressing S1943A and S1943D GFP-NMHC-IIA. These studies support the

hypothesis that myosin-IIA assembly is subject to multiple and distinct regulatory mechanisms in vivo.

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P24-14: FUNCTION AND REGULATION OF Flt-1 IN HUMAN BREAST CANCER

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In addition to its role in angiogenesis, vascular endothelial growth factor (VEGF), produced locally by either tumor or stromal cells, engages VEGF receptors on tumor cells and initiates a signaling response that facilitates survival in the face of apoptotic stimuli, such as tissue hypoxia. The VEGF/placental growth factor (PlGF) receptor Flt-1 is upregulated in many common tumors, including breast, and has been found to mediate VEGF autocrine signaling loops essential for survival in colon carcinoma cells and in neuroblastoma cells exposed to hypoxia. In breast carcinoma, Flt-1 has been identified as a key component of a "poor prognosis gene signature" that is strongly predictive of a short interval to distant metastases. Furthermore, Flt-1 expression, as assessed by immunohistochemistry, has been correlated with a high risk of metastasis and relapse. Collectively, these studies suggest that Flt-1 promotes an aggressive phenotype in human breast cancer. An important issue that arises from these findings is the mechanism by which Flt-1 expression is regulated in breast cancer. Recent evidence has shown that microRNAs (miRNAs), a class of small non-coding RNAs that control gene expression by targeting mRNAs and triggering either translational repression or RNA degradation, are aberrantly expressed in human disease, including human breast cancer. Furthermore, a miRNA expression signature is able to discriminate between normal and breast tumor tissues. Targets of miRNAs down-regulated in breast cancer may include oncogenes or genes encoding proteins with oncogenic functions. Flt-1 has been identified as a putative target of miR-10b, a miRNA which has recently been implicated in breast cancer. miRNAs such as miR-10b may regulate the expression of Flt-1 in human breast cancer and have important implications for tumor progression. Here we report that ectopic expression of a miR-10b mimic in human breast cancer cell lines that lack miR-10b significantly decreases the expression of Flt-1 at the protein level, while a miR-10b mutant mimic with a single base pair substitution in the seed region has no effect, as compared to a non-targeting control. The miRNA mimic is a small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs. Interestingly, expression of miR-10b mimic also inhibited the migration of these cells. These data indicate a potentially important role for miR-10b in regulating both VEGF signaling and migration in breast cancer. Ongoing studies are aimed at confirming these results using a miR-10b expression vector, assessing the expression of miR-10b as a function of breast cancer progression, establishing the function(s) of Flt-1 and identifying novel targets of miR-10b that may influence the behavior of breast cancer cells.

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P24-15: ABL TYROSINE KINASES REGULATE CELL-CELL ADHESION VIA RHO GTPASES

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Deregulated cell-cell adhesion and increased invasion are hallmarks of tumor progression and metastasis. Adherens junctions are calcium-dependent, cell-cell contacts that link neighboring cells via cadherin receptors. Formation and dissolution of adherens junctions are processes of paramount importance during breast cancer metastasis. Cadherin-mediated intercellular adhesion requires dynamic regulation of the actin cytoskeleton, but the pathways that link cadherin signaling to cytoskeletal regulation remain poorly defined. We have identified the Abl family kinases as critical mediators of cadherin-mediated adhesion and have uncovered a novel role for the Abl tyrosine kinases downstream of cadherin-mediated adhesion and upstream of actin dynamics via the regulation of Rho family GTPases. Abl kinases are activated by cadherin engagement, localize to cell-cell junctions, and are required for the formation of adherens junctions. Notably, Abl kinases are required for Rac activation during formation of adherens junctions and also regulate a Rho-ROCK-myosin signaling pathway that is required for the maintenance of intercellular adhesion. Unexpectedly, we have found that Abl kinases also regulate the formation and strengthening of adherens junctions downstream of active Rac, and Abl kinases are components of a positive feedback loop that employs the Crk/CrkL adaptor proteins to promote the formation and maturation of epithelial adherens junctions. Further, we found that Abl kinase activity is required for invasion of metastatic breast cancer cells. Thus, Abl kinase function is required for the regulation of cell-cell adhesion and invasion in breast cancer cells.

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P24-16: FUNCTION OF Smad1 AND Smad5 IN TGF- β STIMULATED MIGRATION

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In breast cancer, the transforming growth factor β (TGF- β) signaling pathway is thought to induce tumor-suppressive and tumor-promoting effects. Numerous studies have determined that the TGF- β type I receptor (a.k.a. ALK5) substrates, Smad2 and Smad3, mediate the tumor-suppressive effects of TGF- β . However, fewer studies have identified the effector proteins that mediate tumor-promoting responses to TGF- β such as migration and invasion. Herein, we report an unexpected role for bone morphogenetic protein type I receptor substrates, Smad1 and Smad5, in TGF- β stimulated migration of a subset of mammary epithelial cell lines. We show that TGF- β induces Smad1 and Smad5 phosphorylation and cell migration in an ALK5-dependent manner as assessed by using an ALK5-specific kinase inhibitor and ALK5 short hairpin RNA (shRNA)-expressing cells. Moreover, results from experiments using a mammary carcinoma line expressing shRNA targeting Smad1 and Smad5 alone or in combination demonstrate that a full migratory response to TGF- β depends on the total level of Smad1 and Smad5 expression. Thus, we propose that TGF- β can stimulate breast cancer cell migration through a non-canonical ALK5-Smad1/5 pathway, and that this pathway may underlie the migratory behavior observed in some clinically relevant breast cancer model systems

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P24-17: PIAS γ MEDIATES NEMO SUMOYLATION AND NF- κ B ACTIVATION IN RESPONSE TO GENOTOXIC STRESS

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The transcription factor NF- κ B is often deregulated in breast cancer cells, exhibiting high basal activity that can be further increased in response to genotoxic anticancer agents. NF- κ B activity is implicated in increasing resistance of cancer cells to chemotherapeutic drugs and radiation-induced death. Protein modification by SUMO (small ubiquitin-like modifier) is an important regulatory mechanism for multiple cellular processes. We previously demonstrated that SUMOylation of NEMO, the I κ B kinase (IKK) regulatory subunit, is critical for NF- κ B activation by genotoxic agents. However, the nature of the SUMO ligase and the mechanisms involved in NEMO SUMOylation remain unclear. Here we provide evidence that a SUMO ligase, PIAS γ , is critical for NF- κ B activation in response to a wide array of genotoxic agents in multiple cell systems, including certain breast cancer cells. PIAS γ interacts with NEMO through an N-terminal domain and promotes its site-selective SUMOylation. Increase or decrease of PIAS γ expression correspondingly increases or decreases NF- κ B activation only by genotoxic agents. Collectively, our findings demonstrate PIAS γ is the critical SUMO ligase for NEMO and this SUMOylation pathway may provide a novel target for development of anticancer agents targeting different human cancer types, including breast cancer.

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P24-18: ROLE OF STAT5A LOSS IN PROGRESSION OF ER α -INITIATED MAMMARY DUCTAL HYPERPLASIA AND DUCTAL CARCINOMA IN SITU

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Introduction: STAT5a is a transcription factor with various functional roles depending on the tissue and its developmental stage. However, the role of STAT5a in breast cancer (BC) is under investigation. Estrogen receptor (ER) α acts as a transcription factor upon estrogen binding. Its role as a growth factor in normal mammary gland (MG) development is well established, and it also plays a significant role in BC development. This research tests further the role of Stat5a in BC by exploiting a new preclinical mouse model (CERM) that develops ER α -initiated mammary ductal hyperplasia (DH) and ductal carcinoma in situ (DCIS) that parallel human disease. To test our hypothesis that loss of Stat5a will alter differentiation, proliferation, and/or survival of mammary epithelial cells with deregulated ER α expression, we will determine if loss of Stat5a alters development of ER α -initiated mammary DH and DCIS.

Results: Abnormal ductal and lobular mammary disease was maintained (p=0.0275) and hyperplastic alveolar nodules (HANs) were present (p=0.0245) at 12 months of age in CERM compared to wild-type (WT) mice. Interestingly, loss of Stat5a in the CERM mice was associated with an increase in abnormal ductal and lobular mammary disease (p=0.0176) and a decrease in HANs (p=0.0031). The MG/mouse weight ratio and ductal length were not altered. ER α -induced abnormal cell proliferation in the MG of CERM compared to WT mice (p=0.0067) was not dependent upon STAT5a. The apoptotic index and cellular localization was unchanged. During normal puberty, estrogen induces first the development and then with progesterone the differentiation of terminal end buds (TEBs) into terminal ductal ends. Results showed an increased percentage of Stat5a-deficient CERM mice as compared to WT mice with TEBs at 2 months of age and TEB-like ends at 4 months of age (p=0.0422). The numbers of TEBs and TEB-like ends were significantly increased at both 2 (p=0.0211) and 4 (p=0.0310) months of age. At 12 months of age, approximately 5% of CERM/Stat5a and CERM/Stat5a^{-/-} mice demonstrated more than 3 TEB-like ends greater than 10,000 μ m². None of the other genotypes studied demonstrated this. This observation suggests that STAT5a plays a role in differentiation of TEBs, which may contribute to the alteration of the development of abnormal mammary disease. A potential in vitro crosstalk between STAT5a and ER α in breast cancer cells has been published. To determine whether ER α and STAT5a can coordinately regulate gene transcription in vivo, cDNA microarray analysis was performed. Sixty-one mapped genes were upregulated in the MGs of CERM mice and downregulated in the MGs of CERM/Stat5a^{-/-} mice. Seventy mapped genes were downregulated in the MGs of CERM mice and upregulated in the MGs of CERM/Stat5a^{-/-} mice. Interestingly, 11 genes were linked to cancer (p=0.001-0.0475). In addition, 9 genes were linked to cellular development (p=0.0036-0.0475), 10 genes to cell cycle (p=0.006-0.0483), and 12 genes to nervous system development and function (p=0.006-0.0475).

Conclusion: Stat5a loss in the CERM mouse model increased the prevalence of abnormal ductal and lobular structures while it decreased the prevalence of abnormal alveolar structures, which is possibly due to an impaired differentiation process. Understanding the implications of STAT5a in different types of breast neoplastic disease and determining the downstream molecular players will help find more specific drug targets.

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GENOMIC INSTABILITY

Poster Session P25

P25-1: IS NUCLEAR STRUCTURE ALTERED IN BREAST CANCER CELLS?

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Alterations in nuclear structure, such as chromatin loops, play a role in normal development and diseased state. Whether such alterations occur in the transformation from normal to breast cancer cells is largely unknown, despite utility of nuclear morphology in breast cancer staging, and observance of changes in nucleoli and perinuclear compartment. Cytogenetic analysis of metaphase chromosomes shows that transformation and progression of breast cancer involves chromosomal alterations, aneuploidy, and genomic instability. As the morphology of the interphase nucleus is altered in breast cancer cells, it is likely that the internal structure of the interphase nucleus is also altered. For the nucleoplasm, organization of estrogen receptor (ER) binding sites was different in different breast cancer cells, indicating underlying structural changes in the interphase nucleus; whether these differences in ER-binding site organization exist between normal and breast cancer cells is unknown. It is hypothesized that changes in nuclear architecture exist between normal and breast cancer cells, which affect chromosomal location, regulatory protein subnuclear localization, and sites of "transcription factories." Analysis of specific components of the nucleoplasm should provide a framework for how the internal organization of the interphase nucleus is altered in breast cancer.

One objective of the research is to assess the distribution of ER-binding sites in normal and breast cancer cells. To detect the ER-binding sites, GFP-tagged ER α and commercial anti-ER α antibodies along with fluorescent secondary antibodies are being used. To optimize immunofluorescence staining and determine which commercial antibodies are suitable for immunofluorescence microscopy in normal and breast cancer cells, immunofluorescence with two commercial anti-ER α antibodies has been performed on ER α breast cancer cell lines, MCF-7 and MDA-MB-231, with or without the ER α ligand, estradiol. Preliminary analysis showed that depending on the source of the antibody, differences in the staining patterns may be observed.

The distribution of RNA polymerase II "transcription factories" is also being assessed in normal and breast cancer cells. Of four commercially available antibodies against the largest subunit of RNA polymerase II, we found two of the four to be suitable for immunofluorescence staining. With one of these two antibodies, our preliminary results showed greater nucleoplasmic staining over cytoplasmic staining for an antibody directed at the hyperphosphorylated C-terminal tail of the largest subunit of RNA polymerase II in MCF-7 cells than in MDA-MB-231 cells.

Further analysis for the potential utility of ER-binding sites, "transcription factories," and chromosomal location as "morphological markers" for distinguishing normal from breast cancer and in breast cancer progression will be performed with normal human mammary epithelial cells and immortalized but non-tumorigenic breast epithelial cells. The current study will assess whether ER-binding sites, "transcription factories," or chromosomal locations are altered in breast cancer.

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P25-2: SIMULTANEOUS DETERMINATION OF EPIGENETIC DNA METHYLATION AND CHROMATIN STRUCTURE IN BREAST CANCER CELLS AT SINGLE-MOLECULE RESOLUTION

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Epigenetic gene regulation is a crucial event in the development of breast cancer and other malignancies. High levels of cytosine 5-methylation (m⁵C) at regions in the genome with a high density of CpG sites, called CpG islands, can promote cancer by inactivating tumor suppressor genes. Conversely, low levels of m⁵C at oncogenes and repetitive elements can respectively lead to gene activation and genetic instability that produce cancer. Similarly, acetylation or methylation of histone proteins that package DNA into nucleosomes can affect DNA binding by transcription factors through alterations in chromatin structure that affect gene expression. We hypothesize that global epigenetic changes in DNA methylation and/or chromatin structure frequently underscore progression to breast cancer. To test this hypothesis, high-resolution genome-wide maps of epigenetic changes in normal and breast cancer cells are needed. Current genomic approaches for mapping m⁵C and chromatin structure are limited in resolution and are assayed via separate population-ensemble techniques. These techniques average away the contributions of individual molecules in experimental samples.

To circumvent these limitations, we have developed a novel single-molecule method that is capable of simultaneously detecting both nucleosome positions and endogenous m⁵C at CpG sites, termed methyltransferase accessibility protocol for individual templates (MAP-IT). In previous studies, we have firmly established that nucleosomes and DNA-bound site-specific factors hinder the accessibility of DNA methyltransferases to their cognate sites. In MAP-IT, the chromatin structure in nuclei isolated

from tissue culture cells is probed by incubation with a DNA methyltransferase that recognizes GpC sites. Patterns of DNA methylation are determined by the technique of bisulfite genomic sequencing in which DNA cytosines are converted to thymines but m⁵C residues are retained. Sequencing molecules cloned from PCR amplicons of interest reveal the methylation status of each GpC and CpG site along an individual DNA strand from a single cell except at GpCpG sites where methylation by both enzymes overlaps. At GpCpG sites, methylation by GpC probe versus endogenous CpG-methylating enzymes can often be inferred by analysis of samples not treated with the GpC enzyme. MAP-IT results will be presented for several genes that exhibit epigenetic alterations in chromatin structure in response to changes in endogenous methylation in normal and breast carcinoma cell lines. These studies are expected to increase our understanding of the relationship between DNA methylation and chromatin structure, which are key regulatory signals in tumorigenesis. Whereas genetic mutations in cancer, for example in the commonly mutated BrCa1 tumor suppressor gene, cannot yet be restored to normal, epigenetic changes are highly amenable to reversal and thereby provide attractive targets for therapeutic intervention in breast cancer.

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P25-3: MAMMALIAN ORTHOLOG OF DROSOPHILA MOF THAT ACETYLATES HISTONE H4 LYSINE16 IS ESSENTIAL FOR EMBRYOGENESIS AND ONCOGENESIS

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Washington University

The mammalian ortholog of the *Drosophila* MOF (males absent on the first) gene product is a histone H4 lysine 16-specific acetyltransferase. MOF belongs to the MYST family of acetyltransferases, which have been associated with acute myeloid leukemia (MOZ), transcriptional silencing in yeast (SAS2 and YBF2/SAS3), interactions with HIV Tat in humans (TIP60), and dosage compensation in *Drosophila* (MOF). Recent studies have shown that depletion of hMOF in human cell lines leads to genomic instability, spontaneous chromosomal aberrations, cell cycle defects, altered nuclear morphology, reduced transcription of certain genes and defective DNA damage response (DDR) to ionizing radiation (IR). hMOF depletion results in loss of H4K16 acetylation which correlates with a decrease in DNA damage-induced activation of ATM and prevents ATM from phosphorylating downstream effectors such as p53 and CHK2. MOF plays an essential role in mammals during embryogenesis and oncogenesis. Ablation of the mouse *Mof* (*mMof*) gene by gene targeting resulted in early embryonic lethality and cell death. Lethality correlated with the loss of H4 lysine 16 acetylation (H4K16ac), and could not be rescued by concomitant inactivation of ATM or p53. Vigorous cellular proliferation is common and essential during both embryogenesis and oncogenesis. Therefore, these two processes may have potentially common chromatin modification signatures characteristic of their proliferation status. hMOF physically interacts with ATM and p53 and in vitro, hMOF binds to and synergizes with p53 to increase histone H4K16 acetylation and target gene transcription. While H4K16ac is known to alter higher order chromatin structure into a relaxed conformation, we will discuss whether there is a correlation between this modification and cellular growth both during embryonic development as well as in the process of tumorigenesis. In comparison to primary cells or normal tissue from breast, all immortalized human normal and tumor cell lines, as well as, primary tumors demonstrated similar or elevated hMOF and H4K16ac levels. Accordingly, MOF over expression correlated with increased cellular proliferation, oncogenic transformation and tumor growth. Thus, these data reveal that the acetylation of histone H4 at K16 by MOF is an epigenetic signature of cellular proliferation common to both embryogenesis as well as oncogenesis and MOF is an essential factor for embryogenesis and oncogenesis.

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P25-4: A MOLECULAR DISSECTION OF TELOMERE STRUCTURE SHOULD LEAD TO IMPROVED BREAST CANCER THERAPEUTICS

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The ideal breast cancer treatment would specifically target breast cancer cells yet have minimal or no adverse effects on normal somatic cells. A recent drug target is telomerase, the ribonucleoprotein that maintains chromosome ends in cancer cells giving them unlimited proliferative potential. Because most normal somatic cells are telomerase negative, telomerase inhibitors are potentially ideal for breast cancer therapies alone or in combination with more standard therapies. The continued development and improvement of telomerase inhibitors will benefit from more detailed data regarding the role of the structure of telomeres and how telomerase activity influences telomere structure. We currently know that telomeres consist of repetitive DNA sequences and protect the unique coding region of each chromosome from disruption due to the end replication problem. The ends of mammalian telomeres form a t-loop

structure that protects the ends of chromosomes from being recognized as DNA damage by cellular damage sensing and repair machinery. The t-loop is formed by a single stranded G-rich 3' overhang that forms a duplex with the preceding double-stranded telomeric region and is stabilized by many telomere binding proteins.

While much has been deduced about t-loops based on genetic and biochemical experiments targeting telomere binding proteins, little is known about the timing or mechanism of their formation and how the regulation of their formation interacts with other aspects of telomere biology. This is due largely to a lack of a facile, sensitive assay that is capable of detecting t-loop presence. Currently, the only method available to assess the presence and structure of t-loops is electron microscopy, which requires extensive sample preparation and a large amount of starting material. A straightforward, cell biology-based assay for t-loops would allow for the routine analysis of t-loop formation and the investigation of t-loop involvement in properties of telomere biology that are pertinent to improving telomerase inhibition therapy in breast cancer. Because t-loops function in part to hide the telomere ends from DNA damage checkpoints, it is possible that cells that undergo replicative senescence may be unable to form t-loops thus leaving their chromosomes exposed to be recognized as broken DNA. Thus, a t-loop assay can be used to determine the effect of t-loop competence on senescence and apoptosis.

Considerable progress has been made in understanding the forces that may stabilize telomeric t-loops and in developing methodology in which t-loop structure is potentially maintained after DNA isolation from cultured cell lines. This approach should allow us to analyze t-loops under a variety of experimental conditions and generate a large amount of important information critical to our understanding of telomere dynamics during DNA replication, aging, and breast cancer treatment with telomerase inhibitors. A more thorough understanding of these essential regulatory mechanisms becomes especially important to the continued use and development of breast cancer therapies involving telomerase inhibitors, allowing us to design more effective telomerase inhibitors that will induce apoptosis in breast cancer cells but not telomerase-negative normal breast epithelial cells.

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P25-5: PIAS γ REGULATES COHESIN-INDEPENDENT CHROMOSOME COHESION DURING MITOSIS

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Accurate chromosome segregation is the crux of mitosis. When this process fails, genome instability and aneuploidy arise, which are hallmarks of cancers, some genetic disorders, and a leading cause of spontaneous abortions. In eukaryotes, the genome is segmented into several chromosomes. This imposes the added complexity of identifying the two copies of each chromosome in order to segregate them to opposite poles during anaphase. Cells overcome this problem by keeping the two copies of a chromosome (sister chromatids) together until anaphase. A model explaining sister chromatid cohesion by catenations that arise during DNA replication was suggested in 1985. However, elegant experiments in yeast implicated a protein-based cohesion system, leading to a model in which the two sister chromatids are trapped by a cohesion ring, which is cleaved to free the two chromatids at the moment of anaphase onset.

Whether this cohesin-based model is required for sister chromatid cohesion and accurate sister chromatid segregation in human cells had not been tested. Here we show that each one of the steps in the cohesin-removal pathway is dispensable for sister centromere separation in human cells. Furthermore, we show that cohesin is largely dispensable for sister chromatid cohesion, indicating that another cohesion mechanism must exist.

In addition, we identify the SUMO-ligase PIAS γ as a regulator of chromosome cohesion since it is required for sister centromere separation in the absence of hSgo1. PIAS γ +hSgo1-depleted cells have cohered centromeres in the absence of centromeric cohesion, but catenations are still present. PIAS γ depletion also induces reduced centromeric localization of Topoisomerase II α , the only known decatenation enzyme, suggesting that cohesin-independent cohesion in PIAS γ +hSgo1-depleted cells might be due to catenations. This provides the first evidence that centromeric catenations are regulated.

We propose an integrative model in which sister chromatid cohesion is due to the cooperative activities of at least two cohesion mechanisms, the cohesion complex and DNA catenations. In this model, accurate chromosome segregation requires the concerted regulation of these systems. By providing the first evidence of regulation of centromeric catenations we have taken a step toward our understanding of the different mechanisms regulating genomic stability.

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P25-6: A FUNCTIONAL GENOMIC SCREEN IDENTIFIES NOVEL GENES WITH ROLES IN THE MAINTENANCE OF GENOME INTEGRITY

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The DNA damage response (DDR) has a critical role in restraining tumorigenesis. Mutations in DDR genes are associated with increased genomic instability and cancer predisposition, particularly in the breast. In agreement with the hypothesis that the DDR serves as a barrier to tumor progression, the DDR is constitutively activated in precancerous lesions. This includes pre-invasive carcinoma in situ lesions of the breast, among others. Aberrant cycles of DNA replication driven by oncogenic stimuli are thought to contribute to genotoxic stress in these early lesions; however, the genetic alterations that create this instability and activate the DDR are poorly defined. Using a functional genomic screen in human cells, we have identified genes that when depleted by RNAi or overexpressed activate the DDR. Consistent with previous reports, depletion of BRCA1 and BRCA1 interacting protein C-terminal helicase 1 (BRIP1) results in DDR activation. The ability of our screen to identify genetic alterations previously demonstrated to challenge genome integrity validates this methodology. In addition to these expected genome maintenance genes, we identified over 200 genes that also promote genetic instability and DDR activation when deregulated. The cellular functions predominantly affected by these genetic disruptions include DNA repair, replication, mitotic regulation, and gene expression. Furthermore, several of these genome maintenance genes have been identified as potential oncogenes or tumor suppressors in cancers. For example, the expression of breast cancer amplified sequence 3 (BCAS3) is elevated in mammary tumors and is correlated with tumor grade and proliferation. Our results indicate that BCAS3 activates the DDR when overexpressed. Secondary screens and functional analyses aimed at understanding the genome maintenance activities of these genes are in progress. The identification and characterization of genes with novel roles in the maintenance of genome integrity will provide essential clues to the cellular processes underlying tumorigenesis. Furthermore, the characterization of these genome maintenance genes has the potential to uncover novel biomarkers that may prove useful in the treatment of breast cancer.

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P25-7: EVIDENCE FOR INTRINSICALLY ELEVATED SOMATIC MUTATION IN HETEROZYGOUS BRCA AND FA MUTATION CARRIERS IN VIVO AND IN VITRO

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The development of human cancer, including breast cancer, is a multistep process driven by somatic mutations and epigenetic events that mimic their effects. Familial cancers, such as the breast cancer syndromes associated with the BRCA genes, are intellectually challenging in that they are recessive at the cellular level, yet dominant at the organismal level, due to the high probability of somatic segregation ("loss of heterozygosity") of the protective wild-type allele. The BRCA genes have been shown to play a role in the process of DNA double-strand break repair, specifically the pathway associated with the hereditary heterogeneous cancer syndrome Fanconi anemia. Therefore, it has been reasonable to presume that the mechanism of cancer predisposition in BRCA heterozygotes has been solely through the somatic generation of DNA repair deficient clones homozygous or hemizygous for the deleterious mutation. There are two widely applied assays for human somatic mutation, at the hemizygous X-linked gene for the purine scavenger enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) and at the autosomal (heterozygous) locus for the MN blood group, glycophorin A (GPA). Using these two assays, we present evidence of elevated bone marrow somatic mutation frequencies in a carrier of a disease-associated truncation mutation in BRCA1 and of elevated spontaneous mutation frequencies in a set of lymphoblastoid cell lines derived from BRCA1 and BRCA2 mutation carriers. These results indicate that heterozygosity for inactivating mutations in these genes confers a detectable predisposition to mutation that could play a primary role in oncogenesis. Moreover, similar phenotypes can be observed in the otherwise asymptomatic carriers of Fanconi anemia of several groups, suggesting that they also are at increased cancer risk. This intrinsic increase in somatic mutation frequency is manifested early but seems to preclude the normal age-associated increase in somatic mutation seen in disease-free individuals, such that the greatest cancer-promoting effect occurs in young people, whereas by age 60, there is no longer any statistical difference between the populations. Therefore, functional assessment of somatic mutation susceptibility and/or DNA repair deficiency could identify (1) individuals at

increased risk of breast and other cancers and (2) individuals who might be susceptible to specific classes of mutagens, such as ionizing radiation, ultraviolet light, or organic chemicals. The first group would benefit from surveillance and chemoprevention whereas the second could minimize the risk by specifically reducing exposure to known occupational, environmental, and medical carcinogens.

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P25-8: DYSREGULATION OF THE UBIQUITIN SYSTEM IN BREAST CANCER

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Chromosomal instability (CIN) is the loss or gain of complete chromosomes. CIN is a hallmark of most solid tumors including adenocarcinomas of the breast. Identifying the underlying mechanisms of CIN in breast cancer and determining whether CIN drives tumor initiation, promotes tumor progression, or simply accumulates in tumor cells as they proliferate remain open questions. A major inducer of CIN is chromosome nondisjunction, the failure to faithfully distribute one copy of each replicated chromosome to both daughter cells during mitosis.

In healthy cells, chromosome transmission errors are prevented by the mitotic spindle checkpoint. This checkpoint ensures that chromosomes do not segregate prior to each chromatid pair associating with a full complement of microtubules and being under the proper tension. Satisfaction of the checkpoint triggers the ubiquitin-dependent degradation of securin and cyclin B. This results in segregation of a full complement of chromosomes toward each spindle pole. The degradation of securin and cyclin B is mediated by UbcH10, a ubiquitin conjugating enzyme, in cooperation with the anaphase-promoting complex/cyclosome (APC/C). UbcH10 is genomically amplified in many breast tumors but whether too much of the enzyme drives chromosome nondisjunction and CIN in mammary cells has not been explored.

In the initial studies of this project, we found that the level of UbcH10 overexpression in breast tumors correlates with tumor grade. Specifically, using matched normal and tumor samples from 8 patients, we found by western immunoassay with an anti-UbcH10 antibody that 4 out of 4 grade 3 tumors dramatically overexpressed the enzyme whereas only 1 of 4 grade 2 tumors did so. This work was done in collaboration with Dr. Kermit Carraway at UC Davis.

We are now focused on introducing excess UbcH10 into normal, human mammary epithelial cells and determining the consequences of this overexpression on the fidelity of chromosome segregation. These studies will test the hypothesis that excess UbcH10 in mammary epithelial cells overrides the mitotic spindle checkpoint and leads to chromosome mis-segregation and CIN. Mammary epithelial cells will be infected with a recombinant lentivirus co-expressing untagged UbcH10 and a histone H2B-red fluorescent protein (H2B-RFP) fusion protein. The H2B-RFP serves as both a visual marker of infected cells and for fluorescent detection of mitotic chromosomes. The influence of elevated enzyme levels on chromosome segregation will be analyzed by live-cell video microscopy and fluorescent *in situ* hybridization (FISH). Together, our tumor sample analysis and the predicted results of our tissue culture studies indicate that UbcH10 represents a legitimate and novel chemotherapeutic target for the treatment of breast adenocarcinomas.

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P25-9: DNA DIFFERENTIATION IN BREAST CANCER BY METNASE

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There is evidence that the human NHEJ DNA repair protein and histone methylase Metnase plays a key role in physically decatenating replicated chromosomes. Therefore, abnormalities in Metnase expression or function could play a role in breast cancer genetic instability, and thereby produce a more aggressive tumor.

While decatenation can take place with Topo IIa alone, the rate of such decatenation is significantly improved by Metnase. Besides Topo IIa, physical mediators of decatenation are not well described. This work defines another such mediator. The finding that an NHEJ repair component assists in decatenation links DNA repair to decatenation. Error-free decatenation and genomic stability may require a proper ratio of Topo IIa to Metnase. Metnase mediates resistance to Topo IIa inhibitors during cancer therapy.

The DNA repair component Metnase plays an important role in enhancing the ability of Topo IIa in untangling chromosomes. Since Topo IIa is a key target of many chemotherapy drugs, targeting Metnase for inhibition using small molecules may

markedly enhance the activity of anti-Topo IIa chemotherapy. Since over-expression of Metnase in tumors may enhance Topo IIa activity, it may mediate chemotherapy resistance.

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P25-10: DNA COPY NUMBER CHANGES IN SENTINEL LYMPH NODES BREAST METASTASIS

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Background: The sentinel lymph node (SLN) is the first node to harbor malignant cells in breast tumors with metastasis, and currently its positivity is an indication for a complete axillary LN dissection. Some genetic markers have been shown to be associated with LN metastasis; however, at the present, none of them can be used either separately or in combination to characterize the SLN metastasis and certainly cannot be used to influence the decision for an SLN biopsy. Therefore, the accurate evaluation of the SLN is essential prior to complete axillary LN dissection. Progress in this area would include the development of more sensitive and accurate methods using molecular markers.

Objective: Considering the clinical importance of the SLN biopsy in the prediction of LN metastasis, the main purpose of this study was to characterize the DNA copy number changes that are present in the SLN metastasis and compare them to the ones present in their corresponding primary breast tumors (PBT) from the same patient.

Methods: Twenty paired specimens of formalin-fixed, paraffin-embedded (FFPE) tissue were obtained from the Hospital Nossa Senhora das Graças, Brazil. The samples were grossly microdissected for the presence of tumor cells and analyzed for DNA copy number changes using chromosomal comparative genomic hybridization (cCGH). A subset of the samples was also analyzed by array-CGH (Agilent platform).

Results: A homogeneous distribution of the number of genomic alterations per chromosome was observed both in the PBT and in the SLN metastatic lesions analyzed ($P > 0.90$). The DNA copy number changes observed per chromosome, as well as the number of gains and/or losses detected in the SLN lesions, were significantly dependent on their incidence in the PBT (regression coefficient test; $P < 0.001$). A non-random distribution of the number of changes was observed for all the chromosomes analyzed, both in the PBT and the SLN groups ($P < 0.001$), respectively. The most frequent changes present in both lesions were gains of chromosomes 19, 16, 1p32-pter, 20, 17, 12q23-qter, 1q22-pter, 1q22-qter, and 18p and losses of chromosomes 13q13-32, 6q13-q23, and 2q22-q34. In the PBT samples analyzed by array-CGH, the findings obtained were consistent with the cCGH data: alterations were detected in the genes located in the areas where gains/losses have been detected by chromosomal cCGH, including, *TOP2A*, *AIB3*, *HOXB7*, *DLX4*, *HER2NEU*, and other genes.

Conclusions: In conclusion, nonrandom chromosome alterations were observed in both primary and metastatic breast lesions in this study. The abnormalities observed in the SLN were comparable to the ones present in the corresponding PBT, indicating that those aberrations are probably retained during the axillary LN metastatic process and suggesting that the genetic alterations that control the development of axillary LN metastasis are likely to be already present in the PBT samples at the time of diagnosis prior to the occurrence of metastasis.

Relevance: The identification of genetic alterations present in the SLN metastasis will be important to detect the early genetic alterations that occur in the breast metastatic process before distant metastasis occur. These alterations can be used as additional molecular markers that in conjunction with the ones currently available can help in the reduction or elimination of the need for invasive surgical procedures and in the prediction of breast cancer metastasis and future recurrence.

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P25-11: ENFORCED OVEREXPRESSION OF Sp17 PROMOTES ANEUPLOIDY AND CENTROSOME AMPLIFICATION

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Sperm protein 17 (Sp17) (gene name *SPA17*, NM_017425) was initially described as an immunogenic protein exclusively expressed in the testes and spermatozoa. More recent studies have demonstrated that Sp17 is expressed at low levels in some normal somatic tissues and exhibits moderate to high-level expression in a range of malignant cells, including breast cancer. Although Sp17 is frequently overexpressed in tumors, the function of this protein in somatic cells is poorly understood, and its role in neo-

plastic cells has not been reported. To investigate the potential role of Sp17 in tumorigenesis, we transduced the immortalized, nontransformed rodent epithelial cell line, RK3E, and the human breast epithelial cell line, MCF10A, with a pBABE retroviral vector encoding Sp17. Western blot analysis confirmed expression of Sp17 in the Sp17-transduced, clonally derived cell lines; Sp17 expression in nontransduced and vector-only transduced cell lines was below the level of detection by western blot. Forced overexpression of Sp17 induced hallmarks of aneuploidy in both RK3E and MCF10A cells, including frequent multinucleation and increased DNA content as measured by flow cytometry. Karyotype analysis demonstrated a chromosome complement approaching tetraploidy in Sp17-overexpressing cells whereas parental and vector control-transduced cells maintained a stable chromosome complement. Sp17 overexpression in these cells was also associated with centrosome amplification as well as abnormal mitotic figures with multipolar spindles. These characteristics are common features of malignancies. Further, while the growth rate of Sp17-overexpressing MCF10A and RK3E cells in two-dimensional cultures was slowed compared to vector-transduced and parental cells, Sp17-overexpressing MCF10A cells produced larger and poorly organized acini in three-dimensional cultures compared to controls. Collectively, these findings suggest that Sp17 overexpression may contribute to chromosome instability in tumors and represent the first evidence that Sp17 may play an active role in tumor development.

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P25-12: ALLELE IMBALANCE (AI) OR LOSS OF HETEROZYGOSITY (LOH) IN NORMAL-APPEARING BREAST EPITHELIUM AS A NOVEL MARKER TO PREDICT FUTURE BREAST CANCER

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Background: Predicting breast cancer development remains challenging. AI, or LOH, is a DNA abnormality that is common to almost all breast tumors. We had shown that AI in histologically normal breast epithelium [terminal ducto-lobular units (TDLU)] is strongly associated with cancer risk (Larson et al, *J. Clin. Oncol.* 2005). This led to the hypothesis that women who have AI in their TDLUs are at an increased risk for breast cancer. This study tests that hypothesis by examining TDLUs from benign breast biopsies of women in the Nurses' Health Studies-Benign Breast Disease (NHS-BBD) nested case-control study. The NHS-BBD study consists of a subset of women enrolled in the NHS. Cases are women with breast cancer diagnosed by June 1, 1995, with a previous benign breast biopsy and available pathology specimens. Controls are women matched for age and year of benign biopsy, who had not been diagnosed with breast cancer at the time the case was diagnosed, and available pathology specimens. Baseline information and clinical follow-up are available for all subjects.

Methods: Cases (n=44) and controls (n=44) were women 35–55 years of age at the time of benign biopsy whose biopsies lacked atypia and who had no family history of breast cancer. 10- μ sections were cut from paraffin blocks, and TDLUs were removed by laser microdissection. DNA was extracted, and 10–20 ng were used per PCR. AI was determined by a fluorescent, capillary electrophoresis-based detection system (Applied Biosystems 3100) with a standard cutoff (reproducible 33% change in peak height). The microsatellite panel is being optimized to add markers with high heterozygosity and small amplified fragments. AI analyses were performed blinded to case-control status.

Results: A total of 11 subjects have been studied. Seven of 11 could be analyzed, generating 38 TDLUs (range 3–8/subject). (Two subjects had degraded DNA, and 2 had insufficient TDLUs). The 38 TDLUs were examined with the current marker panel consisting of 9 markers on 7 chromosome arms (1q, 7q, 8p, 11q, 16q, 17p, and 17q); a total of 211 heterozygous sites could be scored. Six of the 211 (2.8%) sites, from 4/7 (57%) subjects, demonstrated AI. The mean number of AI per TDLU was 0.158. These rates resemble those found in our previous study in which women with no increased cancer risk versus those with cancer (or high cancer risk) had a rate of AI = 0.6% versus 1.5%; the proportion of women with any AI = 28% versus ~ 82%; and the number of AI per TDLU = 0.087 versus ~ 0.170.

Conclusions: Despite the age of the paraffin blocks used in the current study, AI could be analyzed in histologically normal breast epithelium from benign breast biopsies of women in the NHS-BBD study. Initial results demonstrate that the rate of AI, the proportion of subjects with AI, and the number of AI per TDLU are consistent with those found in our earlier study. This suggests that analysis of the remaining subjects, and subsequent unblinding of cases and controls, will help to elucidate whether AI in normal epithelium can predict future development of breast cancer.

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P25-13: MUTATIONS IN THE CENTROSOMAL COMPONENT PERICENTRIN-370 IN BREAST TUMOR SAMPLES

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Breast cancer is characterized by an increase in the number, size, and microtubule nucleation capacity of the centrosomes. Centrosome amplification and increased size correlate with aneuploidy and chromosome instability. Mutations in the tumor suppressor protein p53 correlate with the increased nucleation capacity of the centrosomes. However, the cause of the increase in size and number of centrosomes is not known. We cloned and characterized a novel centrosomal component, pericentrin-370, from a breast carcinoma cell line where it is overexpressed. We found, fortuitously, that this cDNA contains a frameshift mutation that causes truncation of the protein, resulting in loss of the C-terminal, calmodulin-binding, regulatory domain. Similar mutations in a fungal ortholog are dominant and cause loss of calmodulin-dependence. We hypothesized that truncation of pericentrin-370 with loss of the calmodulin-regulatory domain accentuates centrosomal dysfunction. We have now surveyed 22 breast cancer cell lines and 97 breast tumor samples for similar mutations. We will report our findings.

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P25-14: TRANSFORMING GROWTH FACTOR- β INDUCES APOPTOSIS IN GENOMICALLY UNSTABLE CELLS

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Transforming growth factor β 1 (TGF β) plays an important but complex role in breast cancer. Women with "low signaling" TGF β 1 gene polymorphisms have a 2-fold greater risk for late onset breast cancer, but women with 'high' TGF β 1 polymorphisms are either at increased or decreased risk depending on their age at diagnosis. We have shown in mouse mammary gland that autocrine TGF β regulates mammary epithelial proliferation and DNA damage responses in response to ionizing radiation, which in turn rapidly activates TGF β . Radiation increases the risk of human breast cancer. Radiation-induced genomic instability, which is evidenced by non-clonal chromosomal aberrations or other deleterious events in the progeny of irradiated cells, is thought to contribute to cancer risk. Because of its role as a tumor suppressor, we hypothesized that TGF β would either prevent or suppress genomic instability (GIN) in irradiated human mammary epithelial cells (HMEC). Although addition of TGF β (500 pg/ml) did not prevent GIN, measured by centrosome aberrations, tetraploidy or aneuploidy, in the first three days following radiation exposure, was TGF β significantly suppressed radiation-induced GIN in long term culture. Furthermore, TGF β inhibition increased GIN following radiation compared to controls. We further noted that TGF β addition had the unexpected effect of reducing the levels of spontaneous centrosome aberrations, tetraploidy and aneuploidy, which suggests that TGF β levels under standard culture conditions are suboptimal. Together, these data supported our hypothesis that endogenous TGF β mediates the survival of genomically unstable epithelial cells that occur spontaneously or following radiation exposure. Consistent with this, TGF β treatment significantly increased (p<0.03) apoptosis in HMEC 48 to 72 hours post-IR. Moreover, TGF β specifically increased apoptosis in cells with markers of GIN. The role of TGF β in determining the fate of epithelial cells that become genomically unstable, either spontaneously or as a result of carcinogen exposure, demonstrates tissue-level surveillance of genomic integrity as an additional mechanism of tumor suppression.

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P25-15: LOSS OF PIN1 ACCELERATES GENOMIC INSTABILITY IN MOUSE EMBRYONIC FIBROBLASTS

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Background and Objectives: Pin1 is a conserved peptidyl-prolyl isomerase that specifically recognizes phosphorylated serine/threonine followed by proline motifs (pS/T-P). By catalyzing the cis-trans isomerization of pS/T-P bonds, Pin1 can promote conformational changes in its target phosphoproteins and thereby influence protein stability and/or function. Pin1 has been shown to regulate several proteins that are important in cell cycle progression and breast cancer, including c-Myc, c-Jun, cyclin D1, cyclin E, and p53. Interestingly, the levels of Pin1 have been found to be upregulated in a number of breast tumor specimens, which has prompted further research on the role of Pin1 in breast cancer. For example, Pin1 ablation from mice on a mixed genetic background was shown to protect them against Ras- or Neu-induced mammary tumorigenesis. Although these studies suggest a tumor-promoting role for Pin1 in breast cancer, additional evidence suggests Pin1 can act as a condi-

tional tumor suppressor. Specifically, mouse embryonic fibroblasts (MEF) prepared from Pin1^{-/-} mice on a C57BL/6 isogenic background are sensitized to Ras-induced transformation. Though differences in genetic background provide a plausible explanation for these contradictory findings, further investigation is required to elucidate the precise role for Pin1 in breast cancer.

To determine how Pin1 is involved in breast cancer, a more thorough understanding of Pin1's function in nontransformed cells is important. Previous research using Pin1^{-/-} C57BL/6 MEF led to the finding that Pin1 is a negative regulator of c-Myc and cyclin E. Since the deregulation of these oncoproteins is known to correlate with genomic instability and affect cell cycle progression, the objective of this study was to assess the role of Pin1 in these processes.

Methodologies: Genomic instability in Pin1^{+/-} and Pin1^{-/-} MEF was evaluated by quantifying the percentage of micronucleated DAPI-stained nuclei under fluorescence microscopy. Cell cycle progression and aneuploidy were assessed by the flow cytometric analysis of ethanol-fixed and propidium iodide (PI) stained cells.

Results and Conclusions: The loss of Pin1 from primary and p53-dominant-negative (p53^{DD}) expressing MEF led to an increased number of cells with micronuclei. Furthermore, Pin1 deletion accelerated the induction of aneuploidy associated with the overexpression of p53^{DD}. These findings suggest Pin1 plays a role in maintaining the integrity of the genome perhaps through its ability to regulate c-Myc and cyclin E. Interestingly, cell cycle analysis showed that primary Pin1^{-/-} MEF stall in G1 and S-phase, which is consistent with previous research showing the deregulation of cyclin E leads to impaired DNA replication. This study provides evidence that Pin1 can function as a conditional tumor suppressor. Since Pin1 seems to have this dual nature in terms of its role in cancer, perhaps Pin1 could prove to be a useful therapeutic target in breast cancer but only in the appropriate genetic context.

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P25-16: ASSESSING THE EXTENT OF ALLELIC IMBALANCE IN HUMAN BREAST TISSUE USING A MULTIPLEX PCR SYSTEM

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Purpose: Genomic instability can generate chromosome breakage and fusion randomly throughout the genome, frequently resulting in allelic imbalance (AI), a deviation from the normal 1:1 ratio of maternal and paternal alleles. AI reflects the karyotypic complexity of the cancer genome; thus, it is reasonable to speculate that tissues with more sites of AI have a greater likelihood of having disruption of any of the numerous critical genes that cause a cancerous phenotype. For this reason, we previously developed a robust method to assess the extent of AI that provides a quantitative comparison between samples that can be applied to a variety of frozen and archival tissues. The method does not require matched normal tissue, requires little DNA (~1 ng) and uses commercially available reagents, instrumentation, and analysis software. Here, we evaluate the hypothesis that the mean number of sites of AI in human breast tissue increases as a function of breast disease progression.

Materials and Methods: AI was determined using a multiplex PCR reaction, based on the Applied Biosystems AmpFISTR[®] Identifier multiplex PCR system, that amplifies 16 non-breast cancer related, microsatellite loci from independent locations in the genome. A total of 753 independent specimens of human breast tissues were analyzed, including 62 histologically normal tissues from reduction mammoplasty, 24 specimens with benign fibrocystic changes, 10 atypical ductal hyperplasias (ADH), 122 ductal carcinomas in situ (DCIS) and 535 invasive carcinomas (Stages I-IIIa). Non-parametric Rank Sums Tests were used to evaluate the relationships between the number of sites of AI and breast tissue type.

Results: The mean number of sites of AI was 0.31 in histologically normal, 1.38 in benign fibrocystic changes and 1.00 in ADH specimens. As compared to the histologically normal group, both the benign fibrocystic changes and ADH groups showed a significant increase in the number of sites of AI (p=0.0002 and p<0.0001, respectively). The mean number of sites of AI was 2.63 in 27 specimens of DCIS, 3.24 in 104 Stage I tumors, and 2.84 in 32 Stage IIA tumors. All groups were statistically different when compared to the histologically normal group (p<0.0001 for each). We validated these findings in a larger population-based breast tumor cohort comprised of 494 tumor specimens. The mean number of sites of AI was 3.03 in DCIS, 3.08 in Stage I, 2.98 in Stage IIA, 2.92 in Stage IIB, and 3.50 in Stage IIIA. All categories were statistically different from the histologically normal group (p<0.001 for each). Interestingly, in both tumor cohorts, there was no difference in the number of sites of AI in DCIS compared to the invasive groups or between the invasive groups.

Conclusions: The level of genomic instability assessed in unlinked loci increases along the continuum of breast disease between histologically normal, benign lesions to DCIS. However, there is no difference in the number of sites of AI between DCIS

and invasive carcinomas, as well as between the different stages of invasive carcinomas. These findings suggest that DCIS lesions have the same extent of genomic instability as invasive carcinomas; therefore, intervention should occur before the in situ stage of breast disease.

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P25-17: TREATMENT WITH A TELOMERASE INHIBITOR IN BREAST CANCER CELL LINES CAUSES A TIME DEPENDENT DECREASE IN TELOMERE DNA CONTENT

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Telomeres, specialized protein-nucleic acid structures, stabilize chromosome ends and prevent them from being recognized by the cell as DNA double-strand breaks, thereby preventing degradation and recombination. However, telomeres can be critically shortened, and thereby become dysfunctional leading to the accumulation of genomic instability and promoting tumorigenesis. Since telomere length limits the proliferative capacity of cells, telomere length maintenance, while poorly understood, is an important determinant of breast cancer progression. Telomere length maintenance involves a number of telomere-associated proteins including: telomere repeat binding factor 1 and 2 (TRF1, TRF2), TRF1 interacting nuclear factor 2 (TIN2), protection of telomeres 1 (POT1). In addition, telomere elongation can occur in cancerous cells due to the presence of the reverse transcriptase telomerase. Inhibition of telomerase leads to a decrease in telomere length and possibly changes the expression of the telomere-associated proteins. To examine the effect of telomere length changes on the telomere-associated proteins, a potent telomerase inhibitor N,N'-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylene-tetracarboxylic diimide (PIPER) was used to artificially inhibit telomerase in telomerase positive breast cancer cells. PIPER treatment has previously been shown to inhibit the function of telomerase. Thus, it is reasonable to assume that the inhibitory effect of PIPER on telomerase could lead to gradual telomere attrition in a cell line with otherwise stable telomere lengths.

Cultured breast cancer cell lines; MDA MB 231, MCF7 and MCF10 2A were maintained in the presence of PIPER. Cytotoxicity data was collected over the range of 1-100 µM PIPER for 48 hours. Long-term cultures were maintained at 5 µM PIPER. Cell pellets (n=4) were collected both from treated and untreated cell cultures before treatment and over 30 days of PIPER treatment. Proliferation rates were calculated for each cell line in the presence and absence of PIPER. Telomere DNA content (TC), a proxy for telomere length, was assessed in the long-term PIPER treated cultures and controls using slot blot titration assay. Additionally, mRNA levels of the telomere associated proteins, TRF1, TRF2, TIN2, POT1, and telomerase were assessed by quantitative real-time PCR.

The cytotoxic effect showed a dose dependant increase with increasing PIPER concentration, even at low concentrations. However, greater than 90% of the cells survived treatment with 5 µM PIPER and this treatment did not show a significant effect on cell proliferation. To determine if telomeres were shortened due to treatment with PIPER, breast cancer cell lines were treated for 30 days. Cells collected from the non-treated controls showed no significant change in TC. However the PIPER treated cells showed a time dependant decrease in TC. Measurement of the effect of decreased TC on the expression levels of the telomere-associated proteins in ongoing. PIPER treatment rapidly reduces the telomere content in breast cancer cell lines.

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P25-18: CHECKPOINT1 KINASE (CHK1) IS A CRITICAL GATEKEEPER PREVENTING GENOMIC INSTABILITY AND BREAST CANCER

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Chk1 is an evolutionarily conserved serine/threonine protein kinase required for DNA replication and cell-cycle checkpoints in the presence or absence of genomic insults. Moreover, a Chk1-dependent spindle and DNA damage checkpoint at mitotic exit has been identified using transformed mammalian cell lines. Previous studies have shown that Chk1 is sequestered in the cytoplasm in PTEN-deficient primary breast carcinomas leading to increased genomic instability. Due to the multiplicity of detrimental effects observed following Chk1 depletion, its function in mitosis in non-transformed mammalian cells and its role as an essential tumor suppressor have not been well characterized. Interestingly, we observed phospho-Chk1 expression and distinct localization patterns during unperturbed mitosis in both murine and human cell lines using

immunostaining and Western blot analysis. Using two complementary techniques, microinjection and antibody-transfection, we abrogated Chk1 function specifically during mitosis using two specific anti-Chk1 antibodies. A failure of cytokinesis confirmed by time-lapse microscopy was observed when Chk1 function was disrupted in mitotic cells. Chk1 mice revealed similar mitotic defects *in vivo*. Since disruption of Chk1 function led to cytokinesis failure, we hypothesized that Chk1 might be regulating the activity of key proteins necessary for proper mitotic exit. Accordingly, we identified Cdc14B, a human ortholog of yeast fourteen early anaphase release (FEAR)/mitotic exit networks (MEN) pathways, as a potential mitotic substrate for Chk1. We demonstrated that Chk1 kinase directly interacted with Cdc14B in pull-down assays and was able to phosphorylate Cdc14B *in vitro*. Functional studies using Chk1 antibody transfection, displaced Cdc14B from late mitotic structures resulting in chromosome lagging during anaphase and cytokinetic defects. Thus, the levels and activities of these multifunctional cell cycle regulators appear to be critical to prevent failure of anaphase and cytokinesis, leading to the formation of binucleated progeny, and initiation of genomic instability and cancer. Unpublished results from our laboratory using mouse models demonstrate that parity-induced haploinsufficiency of Chk1 can give rise to mammary adenocarcinomas.

Therefore, we hypothesized that Chk1 heterozygosity may initiate pre-cancerous DNA lesions in multiparous mammary gland. Unexpectedly, we found that pups of Chk1 female mice died on day 1 of lactation. Mammary biopsies and histology of Chk1 glands showed a significant decrease in milk-producing lobulo-alveoli as compared to wildtype counterparts. No developmental defects were reported initially in Chk1 by two independent groups in their published studies characterizing germline Chk1-null mice, which were early embryonic lethal. Therefore, studies are under way in our laboratory to characterize in detail the defects during mammary gland morphogenesis in the Chk1 mice before undertaking future parity-induced tumor studies. Chk1 kinase has been proposed as a potential anticancer drug target along with traditional radiotherapy. Since Chk1 levels are critical for survival and homeostasis of genome stability in proliferating cells, a detailed study of multiple roles of Chk1 kinase in normal as well as tumor cells is critical for designing effective Chk1 inhibitors as potential therapeutics for the treatment of breast cancer.

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GENOMICS AND PROTEOMICS I

Poster Session P26

P26-1: SERUM PROTEOMIC BIOMARKERS FOR BREAST CANCER IN AFRICAN AMERICAN WOMEN

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African American (AA) women are less likely than Caucasian women to have breast cancer but are more likely to die from it. Recent research has indicated that breast tumor biology in AA women is different from that in Caucasian women. AA women are more likely to be diagnosed with breast cancer at an earlier age and with more aggressive form of the disease, characterized by higher grade and negative estrogen and progesterone receptor status. The purpose of this study was to profile the serum proteins from healthy and cancer patients to identify differentially expressed proteins between the two groups that could serve as biomarkers, and complement the LC/MS data to the protein expression data obtained previously from 2D-DIGE/MS analysis on similar type of samples. This pilot study included six AA women with breast cancer and six healthy AA controls. The mean age of women with breast cancer was 47. More than 50% of subjects with breast cancer were estrogen and progesterone receptor negative. We expected that changes taking place in the mammary gland prior to the appearance of breast cancer will be reflected in proteins that will appear in the blood.

Methods: The serum samples were processed on IgY12 (Beckman) antibody column to remove the top 12 proteins. The flow-through fraction was digested using trypsin for LC/MS analysis. The protein digest serum samples were analyzed in triplicates on a high performance LTQFT (Thermo Electron) mass spectrometer coupled to an online Surveyor LC system equipped with an autosampler. Samples were loaded onto a trap column using sample pump. MS pump was used for eluting the peptides onto an analytical column for further separation and online nano-ESI LC/MS/MS analysis. The raw MS data files were imported into DeCyderMS (GE Healthcare) module and data were processed for peptide/protein expression analysis.

Results: DeCyderMS analysis revealed significant altered expression of 215 peptides between the two groups (t-test: ≤ 0.05 ; average fold ratio: ≤ 0.5 or ≥ 2). Most of these peptides were mapped to 28 proteins in the Swiss-Prot database. DecyderMS analysis on all the replicates from each sample is under way to identify the peptide pattern differences between the healthy and cancer samples. In addition to high abundant proteins identified by 2-D DIGE/MS in our earlier study, we identified low abundant proteins such as angiotensinogen precursor, insulin-like growth factor-binding protein complex acid labile, vitamin D binding protein and others. Some of these proteins have been implicated in breast cancer among AA women.

Conclusions: A panel of proteins differed in abundance in the sera of AA cancer patients versus healthy controls; these could serve as biomarkers of early-stage breast cancer. Future efforts will focus on further validation of this panel of biomarkers to gain insight into their role(s) in the etiology of aggressive breast tumors in AA women.

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P26-2: QUANTITATIVE FUNCTIONAL PROTEOMIC ANALYSIS OF THE CELLULAR SIGNALING NETWORK

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To obtain a global view of the molecular events instead of individual molecules, we utilize the systems biology approach to monitor the molecular network response. The goal is to apply functional proteomic data to build a mathematical model for simulating the cellular signaling responses in silico and provide direction for more in-depth experimentations and prediction for breast cancer research. Although DNA microarray technology has been well-developed for gene expression profiling, it only reflects the cellular changes at the transcriptional level. Many diseases and responses arise not only due to genetic mutations but are escalated and manifested from sophisticated protein functional alterations. Therefore, a technology that can elucidate ongoing kinase activities and posttranslational modification events is required to generate a dynamic profile for the posttranslational proteome. Reverse-phase protein microarray (RPPM), in conjunction with the emerging Quantum Dots (Qdots) nanotechnology as detection system, is an innovative quantitative proteomic technology developed recently. This technology offers us the large capacity to monitor the time series and dose responses of cells exposed to different types of stimulation and facilitate the complex functional analysis among different signaling pathways. We will present several lines of quality-control experiments to demonstrate sensitivity, specificity, and reproducibility of RPPM. First, comparing with the traditional western blot, RPPM showed 1,000-fold increase in sensitivity. It detected as low as 0.1 pg purified Akt protein compared to 0.1 ng of detection limit in western blot. Moreover, their remarkable signal linearity over serial dilutions makes it a reliable tool for quantitative analysis (Figure 1). Secondly, we demonstrated the specificity of a validated antibody in

Figure 2. Through the joint efforts with National Cancer Institute and M. D. Anderson Cancer Center, there are more than 1,000 validated specific antibodies in different functional modules throughout the cellular signaling network. Thirdly, RPPM showed much superior reproducibility than DNA microarray when the same samples were measured at different time. Lastly, the novel statistical method called "SuperCurve" has been implemented into our data analysis workflow to generate a response curve for each antibody and the quantitative readout from each dilution series. So far, we are capable of applying RPPM to profile the dynamic responses of several signaling pathways, including DNA damage/repair, p53, AKT, MAP kinase, NF- κ B, apoptosis, cell cycle and cell cycle checkpoints, etc.

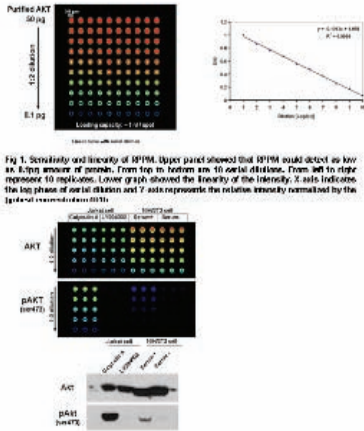


Fig. 1. Sensitivity and linearity of RPPM. Upper panel showed that RPPM could detect as low as 0.1 pg amount of protein. From top to bottom are 10 serial dilutions. From left to right represent 10 replicates. Lower panel showed the linearity of the linearity. X axis indicates the log phase of serial dilution and Y axis represents the relative intensity normalized by the internal cross-reactivity (ICR).

Quantitative Reverse Phase Protein Microarray

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0613 and Department of Energy.

P26-3: IDENTIFICATION OF SUBSTRATES FOR UBIQUITIN-DEPENDENT PROTEOLYSIS DURING BREAST TUMOR PROGRESSION

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Background: Ubiquitination is the covalent attachment of ubiquitin, a small and highly abundant protein onto substrate proteins. Ubiquitination regulates a variety of processes involved in breast tumorigenesis including angiogenesis, apoptosis, and cellular division. A wealth of experimental evidence suggests that the ubiquitin-proteasome system (UPS) is dysregulated in breast tumorigenesis. However, despite much effort, little is known regarding how changes in global cellular ubiquitination activity contribute to breast tumorigenesis.

Objective: We have developed an innovative methodology based on protein microarray technology that can assess ubiquitination activity in complex biological mixtures on a proteome-wide level. We have successfully utilized this technology to identify substrates of specific ubiquitin ligases, cellular fractions, and archival breast tumor extracts (Figure 1). In our research project, we utilize our assay to assess changes in ubiquitination activity that occur during breast cancer progression and attempt to correlate these changes with tumor characteristics, the chemotherapeutic response, and patient survival.

Specific Aim: Assess changes in ubiquitination activity associated with breast tumor progression.

Study Design: Alterations in ubiquitination activity associated with breast tumor progression will be studied by subjecting extracts isolated from primary breast tumor specimens to in vitro ubiquitination reactions on human protein microarrays. Statistical analyses will be used to identify potential associations with tumor characteristics, the chemotherapeutic response, and patient survival. Putative UPS substrates will then be validated using in vitro and in vivo experimental methods.

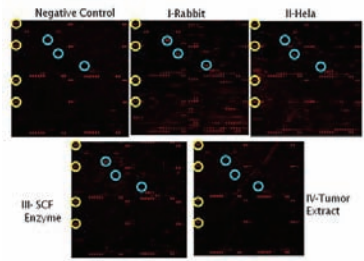


Fig. 2. Specificity of RPPM. Upper panel showed purified Akt protein with serial dilution (0.1 pg to 100 pg). All the samples were spotted in triplicate with 5 serial dilutions. Lower panel is the validation data using Western blot. Cyclosporin A or a phosphatase inhibitor blocks the activation of Akt signaling pathway. pAkt phosphorylation therefore blocks the activation of Akt signaling pathway.

Impact: This research could lead to the identification of novel prognostic markers based on the intrinsic enzymatic activity of breast tumors, which could greatly impact the diagnosis and treatment of breast cancer.

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P26-4: A PROTEOMIC APPROACH TO IDENTIFY PHOSPHORYLATION-DEPENDENT TARGETS OF BRCT DOMAINS

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Background and Objectives: Mutations of tumor suppressor genes such as BRCA1 can cause defects in genome integrity, DNA damage responses, and cellular survival and proliferation. The BRCA1 C-terminal (BRCT) domain, a sequence motif of ~90 amino acids, was first identified in BRCA1. Cancer-associated mutations have been found in the BRCT repeats of BRCA1, suggesting an important role of BRCT domains in regulating BRCA1 activity. In addition, BRCT domains are found in many proteins that regulate DNA damage repair and genome stability, implying a more global role of BRCT domains in genome stability surveillance. Our lab and others have discovered that BRCT domains are novel phosphopeptide binding modules to recruit phosphorylated cellular targets and mediate signaling complex formation. However, the identities of the BRCT domains that are capable of recognizing phosphoproteins and their corresponding in vivo targets remain largely unknown. The overall objective is to systematically identify BRCT-domain-binding phosphoproteins using biochemical and proteomic methods and to examine the functional significance of such interactions.

Methods: Structure-based predictions for phosphopeptide recognition by BRCT domains. To predict whether a particular BRCT repeat may bind phosphopeptides, sequences of human BRCT domains were aligned via the EMBL multiple sequence alignment program. The secondary structures of these BRCT domains were then compared to those BRCT domains whose phosphopeptide-bound structures have been determined. Utilizing Bimolecular fluorescence complementation (BiFC) to identify BRCT domain targets in human cells. BiFC was used to visualize protein-protein interactions in live cells. Two separate proteins are respectively fused to the N- or C-terminal fragment of a fluorescence protein (e.g., YFP). When the two proteins interact, the YFP fragments will be brought to close proximity to form a functional fluorescent complex. To develop the BiFC technology for screening for BRCT-BRCA1 interacting proteins in mammalian cells, we constructed expression vectors. These vectors were designed to encode either the N- or C-terminal half of YFP (YFPn and YFPc, respectively). Next, we generated YFPc-tagged cDNA libraries in our studies.

Results: Candidate phosphopeptide binding BRCT domains in human cells: Based on published structures and our sequence analyses, we have predicted 5 additional putative phosphopeptide-binding BRCT repeats from the human genome. BiFC screens: Interactions between YFPn-BRCA1-BRCT and YFPc-tagged prey proteins would bring YFPn and YFPc to close proximity and allow for the assembly of a functional fluorescent complex. The cells can thus be analyzed by fluorescence-activated cell sorting (FACS) to examine the intensity of the fluorescence and by microscopy for the localization. Indeed, we obtained many YFP-positive cell clones. Preliminary analysis of the clones revealed new BRCA1 binding factors.

Conclusion: In summary, we have found several BRCT domains that can recognize phosphopeptides. We have conducted pilot screens of BRCT domain interacting sequences using BiFC and obtained a number of potential targets for further confirmation and examination. The information obtained from our studies should prove especially useful for the development of new and effective strategies and treatment for breast cancer.

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P26-5: FATTY ACID SYNTHESIS IN BREAST CANCER CELLS IS MEDIATED BY A UPREGULATED ALDO-KETO REDUCTASE FAMILY 1 B10 PROTEIN

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Recent studies have demonstrated that aldo-keto reductase family 1 B10 (AKR1B10), a novel protein overexpressed in human hepatocellular carcinoma and non-small cell lung carcinoma, may facilitate cancer cell growth by detoxifying intracellular reactive carbonyls. This study demonstrates that AKR1B10 is overexpressed in breast cancer tissues and possesses a novel function in tumorigenic mammary epithelial cells (RAO-3), regulating fatty acid synthesis. In these cells, AKR1B10 and acetyl-CoA carboxylase- α (ACCA) are both upregulated simultaneously; and AKR1B10 protein exists in two distinct forms, monomers (~40 kDa) bound to DEAE-Sephacel affinity column and protein complexes (~300 kDa) remaining in flow-through. Interestingly,

co-immunoprecipitation with AKR1B10 antibody and protein mass spectrometry analysis identified that AKR1B10 associates with ACCA, a rate-limiting enzyme of de novo fatty acid synthesis. This association between AKR1B10 and ACCA proteins was further confirmed by the co-immunoprecipitation with ACCA antibody and pull-down assays with recombinant AKR1B10 protein. Intracellular fluorescent studies showed that AKR1B10 and ACCA proteins co-localize in the cytoplasm of breast cancer cells. More interestingly, small interfering RNA-mediated AKR1B10 knockdown resulted in ACCA degradation through ubiquitination-proteasome pathway and an inhibition of fatty acid synthesis in breast cancer cells. These data suggest that AKR1B10 is a novel regulator of the biosynthesis of fatty acid, an essential component of cell membrane, in breast cancer cells, thus being a potential target for the management of this disease.

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P26-6: DIFFERENTIAL PHOSPHOPROTEIN PROTEOME PROFILING OF TAMOXIFEN RESPONSE

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Breast cancer remains the most common malignancy affecting women in the United States. In the majority of breast cancers that are estrogen receptor-positive (ER+), cell proliferation responds to estrogen. Selective ER modulators (SERMs), such as tamoxifen, inhibit cancer growth by competing for binding to ERs. Even in ER+ tumors that initially respond, resistance may develop, rendering tamoxifen ineffective or altering response so that the Tam-ER complex can serve as a growth stimulatory signal. Mechanisms for resistance may include crosstalk between ER and other proliferative signals, including growth factor receptor tyrosine kinase pathways. Determining the mechanisms of tamoxifen-sensitivity and -resistance and defining predictive biomarkers for tamoxifen responses would have great benefit to breast cancer biology and therapy. We are defining signaling states in tamoxifen-sensitive and -resistant breast cancer cells by differential phosphoproteome profiling. Pro-Q Diamond phosphoaffinity resin is used to isolate phosphoproteomes combined with stable isotope labeling, proteolysis, liquid chromatography, electrospray tandem mass spectrometry (LC-MS/MS), and informatics to quantitate differential protein phosphorylation. The method was validated by comparing the cell cycle-regulated (G1 versus G2-phase) phosphoproteomes of yeast and shown to quantitatively retain phosphoproteins and binding partners from cell extracts. Requiring at least two peptides, over 300 proteins were identified with >90% confidence from one gel lane derived from Pro-Q purification of a yeast cell extract. The majority were known phosphoproteins or their binding partners. More than half of the identified proteins have unchanged levels in G1 and G2. Examples of differential profiles include 2.4-fold higher levels in G2 for TMA19 and CCT and 2.2-fold higher levels in G1 for PD11 and YCL042W. Each of the aforementioned proteins was represented by several peptides, all exhibiting equal abundance. These data show that our method is able to identify a small number of differentially regulated proteins from a high number of unchanged proteins. The same approach will be used to identify proteins whose phosphorylation correlates with tamoxifen response, versus estrogen or other SERMs, in MCF-7 breast cancer cells. These studies will provide a signaling profile of tamoxifen action and will enhance our understanding of the correlation between tamoxifen treatment and cancer progression. Finally, information gained is likely to form the basis for more accurate and informed treatment decisions and has the potential to identify new targets to overcome tamoxifen resistance.

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P26-7: IDENTIFICATION OF DNA-BOUND ESTROGEN RECEPTOR COMPLEXES USING A QUANTITATIVE PROTEOMIC APPROACH

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Nuclear signaling by liganded estrogen receptors (ERs) occurs through at least two distinct pathways: (1) direct binding of the receptors to estrogen response elements (EREs) (the "ER/ERE pathway") and (2) indirect recruitment of liganded ERs to activating protein-1 (AP-1)-responsive elements via heterodimers of b-zip transcription factors including c-Fos, c-Jun, and related factors (the "ER/AP-1 pathway"). Although estrogen antagonists can attenuate transcriptional responses through the ER/ERE pathway, they can activate transcription through the ER/AP-1 pathway. This study has focused on defining two mechanistic aspects of ER/AP-1 complexes, namely: (1) the composition of the underlying AP-1 component and (2) the identification of Tamoxifen (Tam)-dependent ER complexes. Specifically, we have used a template-coupled proteomic approach involving isobaric tagging of peptides, called iTRAQ (isobaric tags for relative and absolute quantification), to quantitatively identify the specific polypeptides comprising the AP-1 component of the ER/AP-1 pathway and the Tam-bound ER component of the ER/ERE pathway. The enhancer-

specific factors were isolated from HeLa cell nuclear extract in an in vitro binding assay under conditions similar to those previously shown to support ER-dependent transcription through AP-1 elements. We are currently exploring the role of the identified AP-1 factors in mediating estrogen-dependent responses in the ER/AP-1 pathway at estrogen target genes in MCF-7 and HeLa-ER cells using chromatin immunoprecipitation, reverse-transcription qPCR, and RNAi-mediated knockdown.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0380 and European Commission Marie Curie Outgoing International Fellowship Program (MOIF-CT-2006-40428).

P26-8: QUANTITATIVE PROTEOMICS OF NUCLEAR MATRIX PROTEINS IN NOVEL HUMAN DUCTAL CARCINOMA IN SITU MODEL SYSTEMS

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The earliest identifiable breast cancer lesion, ductal carcinoma in situ (DCIS), is a pre-invasive malignancy. Clinically, tumor grade, size, and the presence of necrosis are used to determine which cases will become invasive, but these have proven ineffective with many cases of low-grade DCIS, which often progresses to invasive disease. With 52,720 women diagnosed with this disease in 2007, a better way to determine which cases need to be treated aggressively is necessary. A subcellular region that holds much promise in this regard is the nuclear matrix (NM). The NM is the structural scaffolding of the nucleus, playing a role in DNA replication, transcription, repair, RNA processing and hormone action. As demonstrated by the nucleus's role as a cellular landmark in the pathology of cancer, NM proteins (NMPs) have shown much promise as biomarkers for cancer. They can be detected in both the serum and urine of patients. We hypothesize that detectable differences exist between invasive and noninvasive DCIS at the protein level, particularly in the nucleus, and these differences are distinct from those demonstrated at the nucleic acid level. This study uses unprecedented DCIS- and breast reduction mammoplasty-derived cell lines generated through a tissue engineering system. We have isolated NMPs from one DCIS line and two normal lines. The DCIS line comes from a 39-year-old woman who had widespread DCIS with an 8-cm ER+ mass. Two different proteomic approaches, difference gel electrophoresis (DiGE) and iTRAQ, are being conducted on the NMPs from these lines. In DiGE, each sample, adjusted for equal protein content, is individually labeled with an electrophilic version of a fluorescent Cy dye, PrCy3-OSu or MeCy5-OSu, mixed, and proteins segregated by 2D gel electrophoresis on a single gel. The two images (Cy3 and Cy5) of the gel are then analyzed by high resolution densitometry and the spots showing differential levels are, robotically, picked as gel plugs, dehydrated, swelled with trypsin, the released tryptic peptides are collected, desalted and, typically, spotted onto a MALDI target. The tryptic peptides are then analyzed by high resolution MS and MS/MS to identify the proteins after comparison of the acquired spectra to theoretical spectra from genomics databases. In iTRAQ, samples are adjusted to equal protein content, individually digested with trypsin, and each sample is labeled with an isobaric reagent that reacts with lysine side chains and the N-terminus. The samples are then mixed and the peptides taken through a multi-dimensional liquid chromatographic segregation with the first dimension being ion exchange chromatography generating 13 fractions from increasing salt concentration, followed by C18 RP-HPLC segregation of peptides from each bump-off into 30 sec fractions on MALDI plates. The peptides are analyzed by MS and high and low energy CID MS/MS. The MS and low energy MS/MS analyses provide identifications as described above, while the high energy CID MS/MS spectra provide relative quantitation, as each isobaric label gives rise to a unique fragment ion in an otherwise "quiet" region of the fragmentogram. It is the ratios of these ions in the m/z 114–120 range that allow quantitative determinations to be made. Although replete analysis is far from complete, several known NMPs have been identified, as well as some unique proteins. Interesting differences between the DCIS and normal samples have also been noted.

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P26-9: CROSS-SPECIES ANALYSES OF GENOMIC CHANGES IN MAMMARY CANCER

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miRNAs have recently been identified as epigenetic elements that have important roles in development, differentiation, apoptosis, and oncogenesis. Altered expression of several miRNAs have been reported in human breast cancers and may be useful in predicting patient prognosis. The functional roles of miRNAs in tumor development

and progression have not been well evaluated. The purpose of this study is to use multiple genetically engineered mouse (GEM) models of mammary cancer as a filter to identify miRNAs whose expression may be evolutionarily conserved in breast cancer. Models studied include MMTV-h-ras, MMTV-her2/neu, MMTV-c-myc, MMTV-PyMT, C3(1)/Tag, transplantable p53 knock-out, and BRCA1-/-; p53. miRNAs that are identified through a cross-species comparison are likely to be functionally important. This study has determined the miRNA expression patterns in multiple mouse models of mammary cancer that are based upon different initiating oncogenic events. Four general patterns of miRNA expression have been identified among the models by hierarchical clustering analyses. A distinct miRNA expression pattern has been identified for MMTV-her2/neu tumors, another for MMTV-myc tumors, whereas p53-/- tumors cluster separately from C3(1)/Tag and MMTV-PyMT tumors. Ongoing analyses are (1) correlating changes in miRNA expression with array CGH and gene expression data from the same tumors arising in the different GEM mouse model systems; (2) determining whether subsets of gene expression patterns correlate with predicted miRNA targets; and (3) comparing changes in miRNA, CGH, and gene expression patterns in the GEM models with those observed in subtypes of human breast cancer.

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P26-10: PREDICTION OF RESPONSE TO CHEMOTHERAPY FOR BREAST CANCER

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Breast cancer recurrence is a major cause of morbidity in patients who develop this disease. Although adjuvant systemic chemotherapy results in significant improvement of clinical outcome in patients with breast cancer disseminated to the lymph nodes, it remains very difficult to predict individual responses to therapy, or long-term survival. The decision to treat lymph node negative patients with adjuvant chemotherapy is presently based on clinical-pathological criteria, but at least 25% of these patients will nevertheless have recurrence and eventually succumb to the disease. These data highlight the need for novel approaches to integration of large data sets including clinical and molecular genetic information to predict individual treatment responses or survival. A related and equally important question is whether increased knowledge of molecular predictors of response could lead to improved methods for sensitization of resistant patients to the beneficial effects of these agents.

We propose to develop predictors based on combinations of gene copy number changes in DNA isolated from fresh tumor biopsies or paraffin sections. There are substantial practical advantages in using DNA in a clinical setting rather than RNA, because of its greater stability and the feasibility of isolating intact DNA from archival tissue blocks for validation of any predictors found. Identification of the specific genes involved in determination of breast cancer sensitivity to anthracycline chemotherapy will then be used to develop novel approaches to induce sensitivity in otherwise resistant patients.

The main barriers to achieving these goals are (a) the availability of sufficient numbers of well-preserved breast cancer samples from patients with extended clinical outcome data and (b) development of analytical and computational tools that can handle the vast amounts of molecular data that can be generated from biological samples and can take account of the complex interactions that underlie biological responses. We propose to circumvent these barriers by using a large set of ~500 breast tumor samples from Valencia, Spain, for which outcome data on treatment responses are available. The computational issues will be addressed using novel algorithms that have been developed at UCSF to construct genetic networks associated with combinations of clinical parameters and genetic information rather than lists of single genes. Using these computational tools and the large number of samples available, we will develop and validate DNA-based assays that are founded on BAC array CGH analysis of tumors that did or did not respond to anthracycline therapy. Candidate genes that may be involved in Adriamycin resistance will be investigated using functional assays in a panel of 55 breast cancer cell lines that have been extensively characterized and profiled at UCSF.

The final product from this part of the project will be a set of TaqMan probes for analysis of specific gene copy number changes in tumors. These will be used to develop a rapid and sensitive assay for likelihood of response to chemotherapy for individual patients. Genes associated with resistance to Adriamycin therapy will be used as the basis for development of methods for sensitization of patients to the effects of these therapeutic agents

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P26-11: DNA METHYLATION AND BREAST CANCER

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The loss of DNA methylation in cancer can lead to genome instability and retrotransposon reactivation, while de novo promoter methylation is associated with tumor suppressor gene silencing. Knowledge of the nature of the methylation abnormalities undergone by cancer genomes is of great importance to cancer research, but currently available methods, such as array-based techniques, often solely examine specific gene promoters and cannot address the methylation status of repetitive elements. We have developed a method for global, unbiased, cost-effective DNA methylation profiling by combining fractionation of DNA according to methylation status with ultra-high throughput DNA sequencing on ABI's SOLiD platform and a new computational pipeline to organize the flood of sequence data. At present we have completed the methylation profile of the breast cancer cell line MCF-7, two primary breast tumors, and two matched normal breast tissues. For these initial samples, we assessed the methylation status for over 66 million CpG sites in the genome, creating one of the largest methylation databases to date. From our analysis, we have detected a large number of methylation changes between normal and tumor samples across the genome including in promoters, repetitive elements, and other genomic regions and are now examining the role these changes may play in breast cancer.

Promoters often become hypermethylated in breast cancer tumors even though the genome as a whole is often demethylated. Using cell hybrid studies to determine whether the demethylation phenotype is dominant or recessive, we have determined that DNA demethylation does not have a common mechanism in cancer. Two hybrid lines showed persistence of parental methylation patterns, which suggests a temporal interruption in methylation maintenance. A third hybrid displayed a dominant demethylation phenotype. Future work will address the mechanism of the dominant demethylation.

While DNA demethylation has been shown to increase genomic instability, it may also have an anti-cancer role. Demethylation may activate a checkpoint mediated by demethylation-induced apoptosis, innate immune system response via the TLR9 receptor, and/or adaptive immune system recognition of cancer-testis neoantigens.

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P26-12: GENOME-WIDE ANALYSIS OF FACTOR RECRUITMENT TO ESTROGEN-REGULATED PROMOTERS IN BREAST CANCER CELLS

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Estrogens, such as 17 β -estradiol (E2), have a well-characterized role in the etiology of human breast cancers. As such, estrogen receptors (ERs) are a major drug target in the treatment of breast cancers. Under the current paradigm for E2-stimulated transcription, ERs are thought to promote the recruitment of Pol II to target promoters thus stimulating the expression of target genes. However, the generality of this pattern of regulation across all E2-regulated genes in breast cancer cells has not yet been determined.

To explore the global mechanisms of estrogen-regulated transcription, I used chromatin immunoprecipitation (ChIP) coupled with genomic microarrays (i.e., ChIP-chip) to determine the localization of Pol II to ~19,000 promoters in MCF-7 human breast cancer cells. I identified ~9,000 Pol II-binding promoters, which is considerably more than previously reported in breast cancer cells. Expression microarray analysis in MCF-7 cells showed that gene expression correlates with Pol II binding at promoters. However, an interesting subset of the Pol II-binding promoters was not expressed under the conditions tested. Analysis of Pol II phosphorylation status suggested that these promoters may contain preinitiation complex (PIC)-associated or promoter-proximally paused Pol IIs.

To determine whether estrogen hormones can regulate the activity of Pol II, I performed Pol II ChIP-chip \pm E2 treatment, coupled with microarray analysis of estrogen-regulated gene expression in MCF-7 breast cancer cells. Surprisingly and in contrast to the "Pol II recruitment" model for E2-regulated transcription, more than half of all E2-stimulated promoters were preloaded with Pol II before E2 treatment. Characterization of these promoters, including an analysis of Pol II phosphorylation state, suggested that the preloaded Pol II is poised in the absence of E2 and moves into the body of the gene upon treatment with E2. To examine further this mode of regulation, I determined the localization of factors involved in the establishment of preloaded Pol II, including NELF, a multi-subunit "negative elongation factor." My results implicate NELF in the establishment and activation of promoter-proximally preloaded Pol II by estrogen signaling.

Post-recruitment regulation of Pol II activity has not previously been associated with E2-regulated transcription. This novel mechanism of transcriptional regulation provides a means by which cells initiate rapid transcriptional responses to estrogens and presents new opportunities in the battle against breast cancer.

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P26-13: FINE-MAPPING AND CHARACTERIZATION OF THE MAMMARY CARCINOGENESIS SUSCEPTIBILITY LOCUS, Mcs5c

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Genetic factors have been estimated to account for 30% of a woman's risk to develop breast cancer. The polygenic susceptibility of breast cancer is thought to be due to rare high penetrance alleles, such as the BRCA1 and BRCA2 mutations, and to a number of lower penetrance alleles that are more common in the population. The objective of this proposal is to use a nonbiased comparative genomics approach to identify a gene or genetic element that plays a role in mammary cancer in the rat and evaluate its potential role as a breast cancer susceptibility element in humans.

The *Mcs5* locus was identified in a Quantitative Trait Analysis (QTL) study. Congenic line analysis revealed three loci within *Mcs5* that have an effect on mammary cancer susceptibility. One of these loci, *Mcs5c*, was previously shown to decrease tumor numbers by 60% and was localized to a 4.5 Mb region of rat chromosome 5. Several additional congenic lines that contain various regions of that 4.5Mb interval have been characterized, narrowing the *Mcs5c* locus to a 300Kb region of rat chromosome 5 devoid of known genes. 5C-11, the smallest resistant congenic line to date shows a 35-40% decrease in tumor numbers (p=0.001). We have identified five potential candidate genes within \pm 600Kb of the *Mcs5c* region: tumor necrosis factor superfamily, member 15 (*Tnfsf15*), tumor necrosis factor superfamily, member 8 (*Tnfsf8*), tenascin c (*Tnc*), pregnancy-associated plasma protein A (*Pappa*), and one unknown mRNA: 8030463A06Rik.

Quantitative real-time PCR was performed on *Tnfsf15*, *Tnfsf8*, *Tnc*, and *Pappa*. No difference in the expression of these genes was detected in the mammary gland of *Mcs5c* congenic rats compared to WF rats. Since the mammary cancer phenotype may not be mammary gland autonomous, we measured expression of these genes in the thymus. One of the four genes, *Tnc*, showed a 30% reduction in gene expression in congenic rats compared to WF controls after DMBA treatment (p<0.01). TNC is an extracellular matrix protein. Further expression analysis suggested *Tnc* expression was also decreased in the ovary of congenic rats. We will verify these findings in independent resistant congenic lines and continue our gene expression analysis in other tissues, such as spleen to more fully characterize how *Mcs5c* may be acting.

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P26-14: HORMONAL INVOLVEMENT IN BREAST CANCER GENE AMPLIFICATION

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Genetic instability and rearrangements, including gene amplification, are a hallmark of cancer. Amplification of the HER2 (ErbB2/Neu) gene, encoding human epidermal growth factor 2, occurs in invasive breast cancer (~25%) and in ductal carcinoma in situ (50%–60%). HER2 amplification and concomitant overexpression of this growth factor promote cancer cell growth, acting as a metastasis-promoting factor. It would be desirable to prevent HER2 gene amplification, thereby moderating the aggressive growth of breast cancer cells. The problem is that no one knows what triggers gene amplification. Our recent research suggested that the trigger may be the steroid hormone estrogen. We propose to test this idea. Cancer is believed to occur after a buildup of somatic mutations or other genomic changes. We wish to ask if a genetic or epigenetic changes might produce novel binding site(s) for the estrogen receptor (ER) near the HER2 gene when it is amplified. The novel ER binding site would be absent in normal cells prior to amplification. Our hypothesis, based on our recent data from a model system, is that the ER interacts with the replication machinery to drive re-replication of the HER2 locus, resulting in DNA amplification.

Our specific aims and the study design are:

1. Map ER binding sites in surgically derived HER2 amplified breast cancer tissue, using chromatin immunoprecipitation (ChIP) with an antibody against ER. The immunoprecipitated DNA will be used as a probe for DNA microarray chips ("ChIP-chip") to screen the human genome for hormone receptor binding sites. We will look for differences in ER binding sites between cancer cells and non-cancer cells from the same patient. The positive candidates will be confirmed by quantitative PCR following ChIP.

2. Map replication origins using short nascent strands as probes for DNA microarray chips. Data analysis will identify replication origins that are near ER binding sites, with special attention given to novel ER sites in the cancer genome. An alternate and/or confirmatory approach is sequential ChIP ("re-ChIP") on chip experiments where DNA is immunoprecipitated by antibodies against ER and against Origin Recognition Complex polypeptide 2 (ORC2), thereby pulling down DNA fragments bound by both antigens.
3. Quantify level of HER2 amplification and identify sites of co-amplification in the genome. DNA will be isolated from the same tissue samples used for specific aims (1) and (2) for use as probes for whole genome SNP arrays to quantify gene copy numbers, thereby identifying regions of amplification. The level of HER2 gene amplification will be quantified and any sites of co-amplification will be determined.

This study will examine if a correlation exists between ER binding at novel sites in the breast cancer genome and juxtaposition with putative replication origins that escape normal cellular controls and re-replicate, leading to DNA amplification. This may provide a new paradigm for hormonal induction of breast cancer via gene amplification, leading to new methods of diagnosis and treatment. Our results will indicate if there are other regions that co-amplify with the HER2 locus in the ER positive, HER2 amplified breast cancer patient samples. Other co-amplified genes, within the HER2 amplicon and/or at other regions, could serve as additional novel targets for therapies similar to the approach of using Herceptin to target HER2.

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P26-15: PRIMEGENS-V2: GENOME-WIDE PRIMER DESIGN FOR ANALYZING DNA METHYLATION PATTERNS OF CpG ISLAND

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DNA methylation plays important roles in biological processes and human diseases, especially cancers. High-throughput bisulfite genomic sequencing based on new generation of sequencers, such as the 454-sequencing system, provides an efficient method for analyzing DNA methylation patterns. The successful implementation of this approach depends on the use of primer search software capable of performing genome-wide scan for optimal primers from *in silico* bisulfite-modified genome sequences. To study the methylation effects in breast cancer, we have developed a method, which fulfills this requirement and conducts primer design for sequences including regions of given promoter CpG islands, as shown in Figure 1.

Our method is capable of designing fragment-specific primer pairs for bisulfite-treated sequences at the genomic scale. The developed method has been implemented using the C and JAVA programming languages. This method is incorporated into the second version of PRIMEGENS (PRIMEGENS-v2). It not only searches for appropriate primers but also checks for nonspecific PCR amplification, the latter of which is unavailable in other software tools. While other software tools typically design primers for one gene at a time, PRIMEGENS can design primers for thousands of genes (fragments) by one run. The software is freely available for academic use at <http://diebig.missouri.edu/primegens/>.

We successfully designed primer pairs for 1,012 query sequences. We randomly picked and synthesized 48 pairs of primers and performed bisulfite PCR using bisulfite-modified DNA in a half 96-well PCR plate. Forty one out of 48 primers generated unique PCR products, and all PCR products are similar in size. The results indicate that our method is efficient and reliable for sequence-specific primers.



Figure 1 Primer design flow chart

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HORMONE RECEPTORS I

Poster Session P27

P27-1: CHARACTERIZING THE TRANSMEMBRANE SEGMENT OF ErbB2 USING SOLID-STATE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MOLECULAR DYNAMICS SIMULATIONS

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The neu oncogene encodes a 185 kD glycoprotein transmembrane receptor (p185neu) with intrinsic tyrosine kinase activity. The receptor has a large extracellular domain of 600–630 amino acids, a single membrane-spanning α -helix domain of about 23 amino acids, and an intracellular domain of about 500 amino acids. It is now established that activation of the neu receptor involves its oligomerization, which in turn leads to receptor activation within the oligomers. However, the involvement of the transmembrane domain in oligomerization is controversial. Sequencing data has demonstrated that oncogenic p185neu differs from c-neu by a single point mutation within the transmembrane region of the glycoprotein. Further studies have also provided evidence that specific interactions between the transmembrane helices of neu can mediate dimerization and receptor activation. We aimed to investigate the structural properties of the transmembrane helix of the neu receptor utilizing solid-state nuclear magnetic resonance (NMR) spectroscopy and molecular dynamics (MD) simulations.

The dynamic properties of neu peptides in lipid bilayers were thoroughly investigated from the lipid perspective using solid-state NMR spectroscopy. One-dimensional and two-dimensional ^{15}N and ^1H - ^{15}N solid-state NMR experiments were conducted on uniformly ^{15}N -labeled neu peptide samples uniaxially aligned along the direction of the magnetic field to determine the structure of neu within the lipid environment. These analyses helped us to define the orientation of the neu peptides with respect to the membrane normal. We also prepared a site-specific isotopic ^{15}N -label at the backbone of the peptide by utilizing solid phase peptide synthesis to determine specific residues involved in dimerization.

To complement the NMR studies, MD simulations studies was conducted to characterize the precise orientation of the transmembrane segment of neu within the lipid bilayer. A membrane-explicit water environment was used in the MD simulations: A POPC bilayer large enough to accommodate the ErbB2 peptide was produced by replicating and then truncating a fully pre-equilibrated $8 \times 8 \times 2$ POPC patch. We are presenting the NMR and MD data for the orientation and conformation of the ErbB2 peptide in phospholipid bilayers. Unoriented spectra of the site-specific ^{15}N -labeled peptide were found to be characteristic of a transmembrane segment for the peptide. The aligned spectra of neu in POPC phospholipid bilayers provided 6 resonance peaks ranging from 185 to 200 ppm. The ^{15}N chemical shift resonances of the transmembrane helix corresponded to a tilt angle of $\sim 13^\circ \pm 4$ in POPC phospholipid bilayers. The tilt angles from the ^{15}N NMR data compared favorably with the molecular dynamic simulation studies. The results provide molecular insight about the orientation of neu in the lipid environments and how the orientation is influenced by protein oligomerization.

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P27-2: STRUCTURAL BIOLOGY OF INTER-DOMAIN FEATURES OF THE NUCLEAR HORMONE RECEPTORS

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The overall objectives of our project are to use macromolecular crystallography and X-ray diffraction to produce the detailed three-dimensional structure of nearly full-length nuclear hormone receptor polypeptides in a series of functionally revealing complexes that include bound ligands and DNA. We will describe a recent strategy that has led us to crystallization of PPAR- γ /RXR heterodimer and how we are planning to continue and adapt this strategy to next define the three-dimensional structure of the estrogen receptor α complex with DNA. The strategy takes advantage of hydrogen-deuterium exchange mass spectrometry to provide helpful clues about the relative stability and flexibility of the receptors in various complexes, using different receptor ligands and DNA sites. The crystallization strategy further takes advantage of assessing multiple DNA sites to help assemble a stable crystal lattice that allows for capturing the protein entities and achieving diffraction to useful resolutions. The analyses of the crystal structure, in the case of PPAR-RXR, more fully reveals how nuclear receptors rely on a series of inter-domain contacts, many previously unknown, to mediate their complex physiology and pharmacology.

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P27-3: NEW SITE-DIRECTED SPIN LABELING TOOLS FOR CHARACTERIZING THE DYNAMIC RESPONSE OF THE ESTROGEN RECEPTOR TO THERAPEUTIC AGENTS

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The estrogen receptor (ER) is an important therapeutic target for the treatment and prevention of estrogen responsive forms of breast cancer. Several crystal structures are available for ER bound to selective estrogen receptors modulators (SERMs); however, the dynamic molecular mechanism of ER action remains unclear. Site-directed spin labeling (SDSL) is a powerful emerging technique for probing specific protein conformational dynamics. We present new SDSL approaches to characterizing the detailed structural and dynamic changes of the ER in response to specific ligands. The initial approach is the standard SDSL method of attaching an EPR-visible nitroxide reporter group to a specific amino acid such as cysteine, the position of which may be controlled by site-directed mutagenesis. We have focused on the helix 12 (H12) domain of the ER that is thought to reposition itself according to the activity of the bound ligand. Labels in this region directly monitor the dynamic response of the ER ligands with different biological activities. To complement this standard approach, the estrogenic ligand may be directly labeled with a nitroxide at the 17a position. By measuring the spin-spin distance between the internal ligand label and a label on the protein, the full dynamic behavior of the receptor response may be mapped out. This also allows direct synthetic control over the ligand activity, since substitutions at the 11 β -position of the ligand have also been shown to change it from a potent agonist to a potent antagonist. The spin-labeled estrogens developed in this work provide novel molecular sensors for detailed and accurate correlation of ligand-induced physical changes in the ER proteins with the biological responses to the ligand. Such tools may be used to rapidly predict the pharmacological character of new ligands, thus providing a rational basis for designing new, more selective anti-estrogens in breast cancer therapy.

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P27-4: IDENTIFICATION OF FOUR NOVEL PHOSPHORYLATION SITES IN ESTROGEN RECEPTOR α : IMPACT ON RECEPTOR-DEPENDENT GENE EXPRESSION AND PHOSPHORYLATION BY PROTEIN KINASE CK2

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Activation of estrogen receptor α (ER α) by phosphorylation is important for ligand-independent activation of ER α and endocrine therapy resistance in breast cancer. However ER α phosphorylation at the eight previously identified sites cannot fully account for endocrine resistance or ligand-independent activation. To determine if additional ER α phosphorylation sites exist, COS-1 cells expressing human ER α were labeled with $[32\text{P}]\text{H}_3\text{PO}_4$ in vivo and ER α tryptic phosphopeptides were isolated to identify phosphorylation sites. Previously uncharacterized phosphorylation sites at serines 47, 282, 294, and 559 were identified by manual Edman degradation and phosphoamino acid analysis and confirmed by mutagenesis and phospho-specific antibodies. Phospho-specific antibodies detected phosphorylation of endogenous ER α in Ishikawa, MCF-7, BT-474, and T-47D cancer cell lines by Western immunoblot. In ER α -dependent reporter gene assays in HeLa cells, mutation of Ser-282 and Ser-559 to alanine (S282A, S559A) resulted in ligand independent activation of ER α . Mutation of Ser-294 to alanine markedly reduced estradiol dependent reporter activation. Similar to reporter assays, endogenous pS2 mRNA was induced by S559A in HeLa cells in the absence of estradiol. Estradiol induction of pS2 was attenuated by mutation of Ser-47 to alanine. Motif analysis and in vitro kinase assays demonstrated that protein kinase CK2 directly phosphorylated recombinant ER α at S282 and S559. S559 represents the first phosphorylation site identified in the extreme C-terminus (F domain) of a steroid receptor and the only ER α site in which alanine mutation induces ligand independent activation. These novel phosphorylation sites represent new avenues for regulation of ER α activity and potential sites for pharmacological intervention.

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P27-5: GANGLIOSIDE-MODULATED CO-LOCALIZATION OF ERBB2, ERBB3, AND PHOSPHATASES IN ERBB3-MEDIATED EVASION FROM KINASE INHIBITOR THERAPY

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Introduction and Relevance: ERBB3 is the preferred substrate and mitogenically most potent heterodimerization partner of the receptor tyrosine kinase ERBB2 (HER2), and ERBB3 provides multiple adapter sites for Akt-driven signaling. ERBB2 is highly overexpressed in many breast cancers, and several kinase inhibitors can inhibit almost all of ERBB2 activity in a sustained manner. However, shifts in the phosphorylation/dephosphorylation (P/D) equilibrium for ERBB3 can completely recover ERBB3 phosphorylation and signal transmission by “effectively getting more mileage” out of the residual ERBB2 activity. The nature of this relatively fast, post-translational adaptation mechanism is not clear but appears to be a significant contributor to resistance to kinase inhibitor therapy. Unless 100% inhibition of ERBB2 can be safely achieved and sustained (a goal that is arguably out of reach now and in the near future), the efficacy of any targeted treatment modalities in ERBB-driven breast cancers will be seriously undermined. The kinase-dead ERBB3 does not lend itself to inhibitors of catalysis and, in contrast to ERBB2, ERBB3 cannot be depleted with geldanamycin-type inhibitors of Hsp90. Ganglioside-type complex lipids have been shown recently to control the recruitment of ERBB2 to raft-type microdomains in the plasma membrane that is known to alter basal activation properties of ERBB receptors, including the much more studied EGFR. For EGFR, shifts in ganglioside types and levels are not only associated with tumor progression, but different gangliosides can directly modulate growth factor receptor properties or alter the colocalization of signaling components, including counteracting phosphatases.

Working Hypothesis: Our working hypothesis is that the relatively rapid shift in the P/D equilibrium of ERBB3 reflects dynamic changes in the extent to which ERBB3 and counteracting phosphatases are coassociated. We further propose that this shift and association is mediated by changes in the raft microdomain environment, specifically the levels and composition of cell-surface and raft-enriched gangliosides.

To test this hypothesis we will:

1. Evaluate if phosphatases and ERBB3 are directly preassociated or found together in microdomains. If so, we will test if the degree of direct preassociation changes during rebound from KI treatment.
2. Evaluate whether the inhibition of ganglioside synthesis and resupplementation of specific core gangliosides influences ERBB3 phosphorylation rebound after KI treatment.
3. If a functional connection exists between general inhibition of ganglioside synthesis and the lifetime of activated ERBB3, we will evaluate shifts in ganglioside composition in response to KI treatment.

The proposed work addresses a fundamental shortcoming of current targeted therapy and integrates two areas of research that are currently largely being studied in isolation, the mechanistic role of the dynamic D/P equilibrium and the lipid microenvironment. This work could identify a new class of orthogonal drug targets that are currently not the focus of attention in breast cancer and that have the potential to boost the efficacy of ERBB targeted kinase inhibitor therapy considerably. It may in the long run also help in establishing a functional linkage between dietary aspects and lipid metabolism and breast cancer control.

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P27-6: ROLE OF THE ERYTHROPOIETIN RECEPTOR IN BREAST CANCER PATHOBIOLOGY

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Background and Objectives: Erythropoietin (Epo) is the prime regulator of red blood cell production. It increases red cell numbers principally by means of an “anti-apoptotic” action on erythroid progenitors. Importantly, Epo also acts on other cells and tissues outside of the hematopoietic system, including endothelium, central nervous system, reproductive system, and gut. Epo receptors (EpoR) have also been identified on breast cancer and other malignant human cells, and in vitro studies from our laboratory and others suggest that these EpoR on breast cancer cell lines may be functional. The presence of functional EpoR on cancer cells is of particular concern since recombinant Epo is used widely to correct the anemia associated with chemotherapy and to improve tumor oxygenation for radiotherapy. This concern was heightened recently when a trial of Epo in breast cancer patients was terminated early due to decreased survival in the Epo-treated group. Given the large number of breast cancer patients who receive Epo therapy, an antiapoptotic effect of Epo on breast cancer cells in vivo would have profound adverse consequences. Therefore, it is of vital importance to determine the functionality of the EpoR on breast cancer cells in vivo.

Methodologies: To test the hypothesis that EpoR plays a role in breast cancer pathobiology, we used RT-PCR to screen a series of human breast cancer cell lines for EpoR expression to identify EpoR⁺ cells. We then employed shRNA technology to knock down EpoR expression (resulting in EpoR⁻ breast cancer cells) and confirmed this using quantitative RT-PCR. We compared to growth properties of these cells.

Results: Our results thus far demonstrate significant variation in EpoR expression levels among breast cancer cell lines tested and that shRNA can downregulate EpoR expression. Knockdown of EpoR resulted in reduced growth. Further results to be presented will demonstrate the effect of altered EpoR expression on tumor formation in immune-deficient mice.

Conclusions: The EpoR appears to play a significant role in breast cancer pathobiology. These results may have profound implications for the management of breast cancer patients and could lead to the identification of EpoR in breast cancer cells as a new prognostic indicator and/or therapeutic target.

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P27-7: A NOVEL UNIDIRECTIONAL CROSS TALK FROM THE INSULIN-LIKE GROWTH FACTOR-I RECEPTOR TO LEPTIN RECEPTOR IN HUMAN BREAST CANCER CELLS

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Obesity is a major risk factor for the development and progression of breast cancer. Increased circulating levels of the obesity-associated hormones leptin and insulin-like growth factor-I (IGF-I) and overexpression of the leptin receptor (Ob-R) and IGF-I receptor (IGF-IR) have been detected in a majority of breast cancer cases and during obesity. Due to correlations between increased leptin, Ob-R, IGF-I, and IGF-IR in breast cancer, we hypothesized that molecular interactions may exist between these two signaling pathways. Co-immunoprecipitation and immunoblotting demonstrated that IGF-IR and Ob-R interact in the breast cancer cell lines MDA-MB-231, MCF7, BT474, and SKBR3. Stimulation of cells with IGF-I promoted Ob-R phosphorylation, which was blocked by IGF-IR kinase inhibition. In addition, IGF-I activated downstream leptin signaling. In contrast to IGF-I, leptin did not induce phosphorylation of IGF-IR, indicating that receptor cross signaling is unidirectional, occurring from IGF-IR to Ob-R. Our results demonstrate for the first time a novel interaction and cross talk between the IGF-I and leptin receptors in human breast cancer cells.

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P27-8: MODULATION OF α CALCIUM AND CALMODULIN DEPENDENT KINASE II SIGNALING BY ESTROGEN RECEPTOR α

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The estrogen receptor (ER) subtypes, ER α and ER β , modulate various signaling cascades in the brain to result in a variety of cell fates including neuronal differentiation. We report here that 17 β -estradiol (E2) rapidly stimulates the autophosphorylation of α -Ca²⁺/calmodulin-dependent kinase II (α CaMKII) in immortalized NLT GnRH neurons, primary hippocampal neurons, and the rat hippocampus in vivo. The E2-induced α CaMKII autophosphorylation is ER α - and Ca²⁺/calmodulin (CaM)-dependent. Interestingly, ER α associates with α CaMKII in a hormone-dependent manner, which attenuates the positive effect of E2 on α CaMKII autophosphorylation, suggesting that ER α plays a complex role in modulating α CaMKII activity. However, it appears as though the activating signal of E2 dominates the negative effect of ER since there is a clear, positive downstream response to E2-activated α CaMKII; pharmacological inhibitors and RNAi technology show that targets of ER α -mediated α CaMKII signaling include extracellular signal-regulated kinase 1/2 (ERK1/2), cAMP response element-binding protein (CREB), and microtubule associated protein 2 (MAP2). α CaMKII autophosphorylation was investigated in vivo, and E2 or PPT administration to ovariectomized female rats significantly enhances α CaMKII activity in the hippocampus after 1 hr or 24 hr of exposure. Functionally, the E2-induced α CaMKII signaling influences neurite outgrowth; E2 treatment of primary hippocampal neurons increases mean neurite length, the number of primary processes, and branching of the processes, all of which are significantly decreased by inhibiting α CaMKII action.

These findings suggest a novel model for the modulation of α CaMKII signaling by ER α , which provides a molecular link as to how E2 might influence brain function. A better understanding of this signaling is required for the development of SERMs that possess beneficial agonist activity in the brain and antagonist activity in breast and uterine tissue to reduce the risk of developing breast or uterine cancer.

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P27-9: IDENTIFICATION OF BIOMARKER FOR ACTIVATOR FORM OF ESTROGEN-RELATED RECEPTOR α IN BREAST CANCER

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Estrogen receptor alpha (ER α) has been implicated as a major contributor to breast cancer progression, with its status serving as a biomarker for disease subtype, patient prognosis, and treatment options. ER α is the major target of endocrine-based therapeutics; however, most cancers acquire resistance to these drugs within 5 years. ER α -negative and drug-resistant breast cancers tend to be highly aggressive, with efficacious long-term therapies not yet available. Thus, identification of novel biomarkers would be useful for better disease prognosis and developing new treatment strategies.

One such candidate is estrogen-related receptor alpha (ERR α), a nuclear receptor whose DNA binding domain shares 70% sequence identity with that of ER α . Both receptors can bind estrogen response elements (EREs) in promoter regions of genes. ERR α can also bind certain extended half-site sequences known as ERREs. Unlike ER α , ERR α does not bind estrogens, functioning, instead, in a hormone-independent manner. ERR α is an independent prognostic factor whose amplified expression in breast cancers correlates with increased incidence of disease recurrence and poor clinical outcome. Studies suggest that ER α -negative and drug-resistant cancers may circumvent dependence on estrogen-modulated growth by activating ERR α -mediated transcription. Thus, genes whose expression are specifically regulated by ERR α could serve as novel prognostic biomarkers for the subset of breast cancers in which ERR α contributes to disease progression. Our goal is to identify and validate ERR α target genes and to test their status as biomarkers of hormone-independent breast cancers.

To identify ERR α -regulated genes, we are performing chromatin immunoprecipitation in tandem with microarray analysis (ChIP-chip) to examine ERR α occupancy in the transcriptional promoter regions of genes present in the mammary carcinoma cell line BT-474. Protein-DNA complexes are formaldehyde-crosslinked, sheared, and immunoprecipitated with antibodies to ER α , ERR α , or RNA polymerase II (Pol II). Purified DNA is amplified by whole-genome amplification and submitted to NimbleGen for hybridization to human promoter arrays. We are focusing on promoters associated with ERR α and Pol II, but not ER α . Preliminary data indicate that ERR α contributes primarily to the regulation of genes involved in metabolism and transcription, results consistent with studies of others. In a complementary approach, we are comparing expression profiles of BT-474 cells with endogenous and knocked-down ERR α expression to further refine our list of ERR α target genes.

Once ERR α -dependent expression of candidate biomarkers has been validated by RT-PCR and immunoblotting, retrospective immunohistochemical analyses of breast tumor samples will be performed to assess correlations of candidate biomarker status with treatment, disease recurrence, and survival. Ultimately, this research will help clarify the transcriptional activities of ERR α and, hopefully, lead to the development of novel prognostic tools for determining best therapeutic options and new drugs for treating breast cancers.

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P27-10: DEVELOPMENT OF A BIOSENSOR FOR IDENTIFYING NOVEL ENDOCRINE DISRUPTING CHEMICALS

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Substantial evidence indicates that endocrine disrupting chemicals (EDCs)—particularly those that interact with the estrogen receptor—may play a role in the degeneration of human and animal health. EDCs can mimic or alter the action of the endogenous hormones, which have been shown to affect the reproductive and endocrine systems of mammals and reptiles. EDCs are found in food, water, air, and consumer products and originate from pharmaceutical, industrial, agricultural, and natural sources. Unfortunately, our lack of understanding of exposure to EDCs limits our ability to evaluate their potential role in the environment. Most chemicals in commerce, and their environmental degradation products, have not been screened to identify EDCs. Furthermore, using currently available technologies, it is extremely difficult to identify the active EDCs in complex estrogenic environmental mixtures, such as wastewater, a major source of drinking water pollution. These limitations impede any serious attempt to evaluate the potential impact of these exposures on health.

We have developed a novel new technology—an estrogen-receptor quartz crystal microbalance (ER-QCM) biosensor—to break through this barrier in identifying EDCs relevant in the environment. The ER-QCM detects estrogenic substances using a genetically engineered construct of the hormone-binding domain (HBD) of the estrogen receptor immobilized to a piezoelectric quartz crystal. The ER-QCM senses ligands that are known to bind the ER (e.g., estradiol, tamoxifen, genistein) and shows no response for non-binding substances such as testosterone and progesterone. Our

ultimate goal is to develop the ER-QCM biosensor as a robust tool for identifying estrogenic activity in complex environmental mixtures and to combine the biosensor with other biophysical methods in order to identify novel estrogenic chemicals in a number of environmental water resources. This presentation will show the steps we have taken in the process of this development.

To produce this biosensor, we immobilized the genetically engineered ER-HBD on the gold surface of a piezoelectric crystal via a gold-sulfur bond. The ER-HBD sensor surface is the unique and critical aspect of this biosensor that permits us to detect estrogen receptor ligands by measuring ligand-induced changes in the rigidity of the protein films. Genetic engineering of the ER-HBD to replace exposed cysteines with serine yields immobilized proteins with uniform orientation and eliminates problems with non-specific protein binding to the surface. We will present the sensing capabilities.

This biosensor methodology will generate a new and much-needed tool for identifying EDCs in complex environmental samples. Identifying the most abundant EDC pollutants will ensure that future toxicology studies can consider these exposures and will focus exposure reduction efforts on these pollutants. We will show how this methodology is far reaching in that it can also be applied to other receptor-ligand systems.

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P27-11: A NON-NUCLEAR ROLE OF THE ESTROGEN RECEPTOR α IN THE REGULATION OF CELL-CELL INTERACTIONS

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In estrogen (E2)-dependent breast cancers, E2 not only promotes the proliferation of cancer cells but also the formation of metastases, which involves the disruption of cell-cell interactions such as adherens junctions. The actions of E2 are usually mediated by the ability of the estrogen receptors (ER α , ER β) to alter gene expression in a hormone-dependent manner. Results in our laboratory demonstrated that ER α physically interacts with the adherens junction proteins α -catenin and β -catenin indicating the possibility for a non-nuclear role of ER α in regulating cell-cell interactions. In support of this hypothesis, we found that in human MCF7 breast cancer cells about 5% of ER α co-purifies with membranes and that changes in the membrane-association of ER α affected the formation of cell-cell interactions. However, using indirect immunofluorescence and co-immunoprecipitations, we were unable to demonstrate direct interactions of ER α with adherens junctions, nor did we detect E2-dependent changes in the formation, stability, or number of junctions. Further analysis of the in vitro interaction of ER α with α -catenin and β -catenin revealed that ER α binds preferentially to α -catenin homodimers. Interaction of these homodimers with the Arp2/3 complex promotes the formation of branched F-actin fibers, which are typical for cells with no or immature adherens junctions. The interaction of β -catenin with α -catenin in mature adherens junctions reduces the concentration of α -catenin homodimers and promotes the formation of parallel F-actin bundles, which stabilize cell-cell interactions. In our experiments, E2 treatment increased the concentration of branched F-actin fibers in confluent MCF7 cells suggesting that E2 prevents the disruption of the α -catenin-Arp2/3 complex by β -catenin. In summary, our results support a nonnuclear role of ER α in the E2-dependent regulation of cell-cell interactions and point to the α -catenin homodimer and Arp2/3 complex as novel targets for regulating the formation of metastases in breast cancers.

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P27-12: A ROLE FOR MEK-INTERACTING PROTEIN 1 IN ESTROGEN RECEPTOR POSITIVE BREAST CANCER CELLS

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Estrogen receptor-alpha (ER) plays an important role in the development and growth of breast cancer. ER-positive tumors are treated with endocrine therapies that target the receptor, but resistance to these therapies limits their efficacy. ER mediates its effects on breast cancer cells via interactions with other cellular proteins such as transcriptional regulators and signaling molecules, and these molecules are potential diagnostic and/or therapeutic targets. We have identified a novel, ligand-dependent interaction between ER and MEK-Interacting-Protein 1 (MP1) in breast cancer cells. MP1 is a scaffold protein that binds to MEK1 and ERK1 and increases ERK activity when overexpressed. We found that overexpression of MP1 increases ER's transcriptional activity and cell proliferation in the presence of both estradiol (E2) and tamoxifen (Tam), a Selective Estrogen Receptor Modulator. The ability of MP1 to increase ER activity was independent of ERK activation, suggesting that MP1 is regulating ER via a novel mechanism. Based on these preliminary results, we hypothesized that MP1

serves as a scaffold protein to organize ER-containing protein complexes, and that the ER/MP1 complex is required for ER function and estrogen regulated proliferation of breast cancer cells.

To test the hypothesis that MP1 is required for breast cancer cell proliferation, we used small interfering (si) RNA to knock-down MP1 expression and examined the effects in the ER-positive breast cancer cell line, MCF-7. Western blot analysis confirmed efficient knock-down of MP1 protein levels in cultures transfected with MP1, but not control, siRNA. Cells were then transfected with control or MP1 siRNA and incubated in the presence or absence of E2. Their ability to incorporate BrdU into DNA was measured after 48 hours as an indicator of cell proliferation. Surprisingly, E2 treatment increased the percentage BrdU-positive cells to the same extent in cultures treated with either control or MP1 siRNA, indicating that normal levels of endogenous MP1 are not required for proliferation in this short-term assay. However, treatment with MP1 siRNA did dramatically alter the phenotype of cells, which rounded up and detached from the plates approximately 72 hours after treatment with MP1, but not control, siRNA. This finding suggests that expression of MP1 is required for long-term survival, proliferation, and/or attachment of MCF-7 cells. We are currently carrying out long-term experiments to examine these possibilities and to more completely characterize the role of MP1 in breast cancer cells.

The development of resistance to endocrine therapies limits their efficacy in the treatment of ER-positive breast cancer. Our experiments to date indicate that MP1 interacts with ER and is required for the normal phenotype and function of ER-positive breast cancer cells. Thus, MP1 or associated proteins may provide new targets for the diagnosis or treatment of this most common type of breast cancer.

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P27-13: THE ER & PR STATUS OF THE ORIGINATING CELL OF ER-NEGATIVE BREAST CANCER

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We hypothesize that ER is absent in the originating cell of ER-negative breast cancer. Although until now it has been technically difficult to test it, we have developed a unique mouse model based on the RCAS-TVA technology that allows us to trace the ER status of the cancer-originating cell. It is now possible to test this hypothesis in experimental mice. We plan to first test whether ER-negative mammary tumors arise from PR-negative or PR-positive normal mammary epithelial cells since PR is a transcriptional readout of ER and since we have all the reagents and mice in place to do the experiment. Next, we will directly test whether ER-negative tumors arise from ER-negative or ER-positive normal mammary epithelial cells in vivo after we have finished generating BAC transgenic mice expressing the Cre recombinase from the ER promoter.

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P27-14: NONSTEROIDAL ANTI-INFLAMMATORY DRUG SULINDAC INDUCES APOPTOSIS BY MODULATING THE SUBCELLULAR LOCALIZATION OF RETINOID X RECEPTOR

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Women regularly taking aspirin and related drugs known as nonsteroidal anti-inflammatory drugs (NSAIDs) have decreased risk of developing breast cancer. Their anti-tumor activity of NSAIDs has primarily been attributed to inhibition of the cyclooxygenase-2 (Cox-2). However, accumulating evidence has shown that Cox-2 inhibition is not the sole basis of their anti-tumor activity. Moreover, the well-known undesired side effects of NSAIDs and Cox-2 inhibitors have discouraged the exploitation of their anti-neoplastic potential. The identification of new molecular target for NSAIDs may make it possible to develop new drugs that are likely to be more efficacious and less toxic. Retinoid X receptor (RXR), a member of the nuclear receptor superfamily, is unique as it mediates not only retinoid signaling but also other multiple signal pathways triggered by various members of the family. This unique property greatly broadens the biological activity spectrum of RXR to the corresponding nuclear receptor-signaling pathways and makes them intriguing and unusual targets for pharmacologic interventions. Structurally distinct compounds including 9-cis-retinoic acid (9-cis-RA), a variety of synthetic compounds such as Targretin/bexarotene, docosahexaenoic acid (DHA), and phytanic acid can bind RXR and act as agonists or antagonists for RXR. Some of them have been successfully developed in clinic to treat cancers and other human diseases. Thus, RXR serves as an attractive molecular target for modulating the growth and apoptosis of cancer cells. The mechanism by which retinoid receptors mediate therapeutic effects of their ligands remains to be illustrated. Like other members of the nuclear receptor superfamily, retinoid receptors act as transcriptional factors, which control the transcription of retinoid target genes.

We recently discovered a new paradigm in cancer cell apoptosis, in which orphan nuclear receptor Nur77 migrates from the nucleus to mitochondria where it interacts with Bcl-2 to trigger cytochrome c release. Migration of Nur77 requires its heterodimerization with retinoid X Receptor, providing an opportunity to modulate the Nur77-Bcl-2 apoptotic pathway by RXR ligands. Here we report that sulindac, an approved nonsteroidal anti-inflammatory drug, exerts its apoptotic effects in breast cancer cells by binding to RXR to activate the RXR-nongenotropic signaling pathway. Sulindac binding inhibits transactivation of RXR homodimer and heterodimer and induces translocation of RXR from the nucleus to the cytoplasm. Inhibition of RXR expression or prevention of its nuclear export suppresses the apoptotic effect of sulindac. In cells that express orphan receptor Nur77, sulindac induces nuclear export of RXR/Nur77 heterodimers and their mitochondrial targeting, which is accompanied by Bax conformational change and oligomerization, resulting in apoptosis induction. Our results define a class of RXR ligands that promote the nongenotropic RXR action and they also suggest a new approach for the development of effective RXR-based medicine.

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P27-15: ABNORMAL MAMMARY GLAND DEVELOPMENT AND GROWTH RETARDATION IN FEMALE MICE AND MCF7 BREAST CANCER CELLS LACKING ANDROGEN RECEPTOR

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Phenotype analysis of female mice lacking androgen receptor (AR) deficient (AR^{-/-}) indicates that the development of mammary glands is retarded with reduced ductal branching in the prepubertal stages, and fewer Cap cells in the terminal end buds, as well as decreased lobuloalveolar development in adult females, and fewer milk-producing alveoli in the lactating glands. The defective development of AR^{-/-} mammary glands involves the defects of insulin-like growth factor I-insulin-like growth factor I receptor and mitogen-activated protein kinase (MAPK) signals as well as estrogen receptor (ER) activity. Similar growth retardation and defects in growth factor-mediated Ras/Raf/MAPK cascade and ER signaling are also found in AR^{-/-} MCF7 breast cancer cells. The restoration assays show that AR NH2-terminal/DNA-binding domain, but not the ligand-binding domain, is essential for normal MAPK function in MCF7 cells, and an AR mutant (R608K), found in male breast cancer, is associated with the excessive activation of MAPK. Together, our data provide the first in vivo evidence showing that AR-mediated MAPK and ER activation may play important roles for mammary gland development and MCF7 breast cancer cell proliferation.

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P27-16: ALTERNATE PROMOTER USE AFFECTS ESTROGEN RECEPTOR PROTEIN EXPRESSION: TRANSLATIONAL CONTROL BY AN UPSTREAM OPEN READING FRAME IN THE ER PROXIMAL-PROMOTER TRANSCRIPT

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Estrogen receptor alpha (ER) expression is a key positive prognostic factor in breast cancer guiding both tumor classification and treatment strategy. ER mRNA is primarily transcribed from two promoters. The resulting transcripts carry identical major Open Reading Frames (ORFs) encoding the same ER protein. Control of ER expression may be affected by differing regulation of RNA expression from the alternate promoters and also by differing levels of ER protein generation from the two transcripts. Our data indicate that the promoter origin of ER alpha transcripts may affect subsequent protein expression.

The two ER transcripts differ in regions upstream of the major ER ORF. The 5' regions of the two mRNA transcripts contain upstream open reading frames (uORFs) encoding potential peptides of 20 and 18 amino acids. The peptides have five C-terminal residues in common. We have addressed the role of the uORFs and their encoded peptides in controlling downstream expression of the major ER ORF.

Expression of green fluorescent protein (GFP) reporter constructs containing upstream proximal-promoter transcript sequences with the first 18 codons of ER fused to GFP was tested in transiently transfected cells. The cells expressed reduced levels of GFP as compared to the pEGFP-N1 parent vector; the effect was dependent on the presence of an intact proximal ER transcript uORF. Only protein expression was affected by eliminating the uORF; RNA levels were unchanged. This indicates the mechanism is translational rather than being an effect of the introduced point mutations on either mRNA stability or transcription.

Eliminating the uORF did not significantly increase expression from similar distal promoter transcript ER-GFP constructs. However, the translational start region of the distal uORF was inherently better at initiating translation than the AUG environment

of the proximal promoter transcript uORF. This was found with both in-frame fusions of the entire proximal and distal uORFs to GFP and with GFP under the control of the two translational start motifs. The data indicate there are regulatory properties suppressing expression from the ER translation start which are specific to the unique regions of the ER proximal promoter transcript and these are likely associated with the proximal transcript uORF peptide product.

We have probed the ER proximal promoter transcript uORF for key features relevant to translational control using mutational approaches in DNA constructs and subsequent determination of the levels of reporter RNA, protein and GFP fluorescence expression. The data indicate that C-terminal regions of the proximal uORF have a prominent role in translational control. A key feature of the encoded peptide is a Tryptophan-Proline motif consisting of a bulky "aromatic" residue linked to a potential helix break, although this is influenced by other residues/regions of the peptide. The elimination of the C-terminal Phenylalanine codon by mutation and deletion results in further reduction in expression of a downstream ER-GFP cassette.

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P27-17: SUMOYLATION OF COREPRESSOR SAFB1: MECHANISM OF ACTION AND BIOLOGICAL RELEVANCE IN BREAST CANCER

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Scaffold attachment factor B1 (SAFB1) was originally isolated due to its ability to bind scaffold/matrix attachment regions of genomic DNA. Subsequently, SAFB1 has also been shown to be involved in RNA processing, chromatin organization, and stress response. Work from our laboratory has shown that SAFB1 can bind and modulate transcription of the HSP-27 promoter and repress activity of ER α . Repression of ER α occurs through an independent and transferable c-terminal repression domain. Further work has indicated that SAFB1 can interact with and repress transactivation of a host of nuclear receptors including PPAR γ and FXR, suggesting a general role for SAFB1 in transcriptional repression. Many proteins associated with transcriptional regulation are post translationally modified by small ubiquitin-like modifier (SUMO). SUMO is a 98 amino acid mature polypeptide that is added post-translationally to target proteins usually within a consensus sumoylation motif, YKXE (where Y is a large hydrophobic residue, K-lysine of modification and X any residue). The process of sumoylation is analogous to ubiquitination. SUMO modification of proteins associated with transcription has generally been associated with transcriptional repression. The mechanism by which this modification leads to transcriptional repression has yet to be defined.

Work by our laboratory has indicated that SAFB1 is modified by sumoylation. SAFB1 contains two consensus sumoylation sites that are conserved in the family member SAFB2 as well as other lower organisms. We have mutated both lysines within the two consensus sumoylation sites to arginines (SAFB1 K2R). Mutation of these sumoylation sites leads to loss of sumoylation and loss of transcriptional repression. Transient transfection ERE-Tk-Luciferase assays showed a loss of repression with overexpression of SAFB1 K2R compared to SAFB1 WT. To examine the effect of SAFB1 sumoylation on its intrinsic repressive capability transient transfection Gal4-DBD assays were performed. Loss of transcriptional repression was seen with SAFB1 K2R-Gal4-DBD expression compared to SAFB1 WT-Gal4-DBD expression indicating an effect of sumoylation on SAFB1's intrinsic repressive function.

The mechanism by which SAFB1 is sumoylated and the extent of the functional consequences of this sumoylation has yet to be determined. Ongoing studies within the

lab are aimed at addressing this question. Studies are focused on determining the SUMO conjugating and deconjugating enzymes responsible for SAFB1 sumoylation. Other work will address the potential for altered protein-protein interactions of SAFB1 dependent on its state of sumoylation and potential for altered cellular localization with sumoylation. In relation to the potential role of SAFB1 sumoylation in breast cancer, DNA from clinical breast cancer specimens is going to be sequenced to look for mutations within the SAFB1 sumoylation sites that may be important in clinical breast cancer specimens.

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P27-18: EVIDENCE FOR UNIQUE MECHANISMS OF ESTROGEN RECEPTOR DEGRADATION ELICITED BY ESTRADIOL AND ICI 182,780 (FULVESTRANT)

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Background and Objectives: Selective estrogen receptor down-regulators (SERDs), such as ICI 182780 (Fulvestrant), comprise a class of drugs that has been developed to inhibit estrogen receptor alpha (ER) activity in breast cancer. ICI is a pure antagonist that competitively binds to the ligand-binding domain of ER. The binding of ICI is thought to reduce the receptor's half-life by increasing protein turnover. However, the exact mechanism of ICI action, including whether receptor degradation is actually required, is currently unknown. Our laboratory has shown that a cytoplasmic ER mutant (cER) lacking a nuclear localization sequence (NLS) is completely resistant to ICI-mediated degradation in C4-12 cells (an ER-negative variant of MCF-7 breast cancer cells).

Methods: MCF-7 breast cancer cells and their ER-negative derivatives, C4-12 cells, were stably transfected with either wild-type ER or cER constructs. Immunofluorescence and confocal microscopy were performed to assess localization of all ER proteins. These cells were also treated for different lengths of time with varying concentrations of both estradiol (E2) and ICI. Immunoblotting was performed to determine levels of ER protein.

Results: Immunofluorescence and confocal imaging of stably transfected C4-12 cells has confirmed distinct subcellular localization of each ER protein. Wild-type ER localizes mainly to the nucleus while cER is exclusively cytoplasmic. As expected, treatment of either wild-type MCF-7 cells (or C4-12 cells expressing wild-type ER) with E2 or ICI caused degradation of ER protein. Treatment of MCF-7/cER or C4-12/cER cells with E2 caused degradation of cER protein. In stark contrast, increasing concentrations of ICI failed to cause degradation of cER. Length of treatment also does not seem to affect ICI-mediated degradation of ER. Thus, ICI and E2 must elicit degradation of ER via distinct mechanisms.

Conclusion: ER is a critical marker for response to antiestrogen therapy. Although treatment with ICI is relatively successful, side effects and the failure to respond in some patients are still severe problems. In addition, indiscriminate degradation of ER results in the loss of its beneficial qualities in bone and the cardiovascular system. A better understanding of the mechanism by which ICI mediates ER degradation may lead to the development of new and better antiestrogen therapies to inhibit ER action with less toxicity and greater specificity.

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ENDOCRINE PATHOGENESIS I

Poster Session P28

P28-1: CONTROL OF LUMINAL TYPE A INTRINSIC SUBTYPE ENRICHED TRANSCRIPTION FACTOR NETWORK BY INSULIN: IMPLICATIONS OF DIABETES ON BREAST CANCER SUBTYPES

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Background: Breast cancer is classified into five intrinsic subtypes based on gene expression pattern; luminal type A, luminal type B, ErbB2/Her-2-positive, basal, and normal-like. Luminal type A and type B represent estrogen receptor α (ER α)-positive breast cancers with luminal type A expressing higher levels of ER α and is associated with better prognosis. Recent studies have identified a specific functional transcription factor network comprising GATA-3, FOXA1, and ER α in normal luminal cells as well as in luminal type A breast cancer that dictates their hormone dependence. Signaling molecules that may disrupt this network and force these cells to acquire hormone-independence are not known. T-bet (Tbx21) has been described as a major negative regulator of GATA-3 activity. As the expression and/or activity of some of the above factors are controlled by insulin, the objective of this study was to investigate whether elevated level of insulin, as evidenced in type II diabetes, alters gene expression pattern in luminal type A breast cancers by interrupting GATA-3:FOXA1:ER α network and thus forcing these cancers to acquire nonluminal phenotype and/or hormone-independence.

Methodologies: The effect of insulin on the expression of ER α , FOXA1, GATA-3, and T-bet was measured in ER α -positive MCF-7 cells by western blot analysis. The effect of T-bet on estrogen-regulated gene expression was measured by transient transfection assays and stable overexpression of T-bet in MCF-7 cells. Publicly available Oncomine database was used to determine the expression pattern of T-bet and its relation to ER α status in primary breast cancers. Immunohistochemistry was used to determine T-bet expression in normal breast and breast cancer.

Results: Insulin induced the expression of T-bet, which was partially reversed by estrogen. In transient transfection assays, T-bet reduced estrogen response element-driven reporter gene expression. ER α and GATA-3 levels were reduced in MCF-7 cells stably overexpressing T-bet suggesting that T-bet reduces GATA-3-dependent ER α expression. Estrogen-inducible expression of estrogen target gene GREB-1 was lower in T-bet overexpressing cells compared to parental cells. Importantly, ER α -negative breast cancers showed higher T-bet expression suggesting mutual antagonism between ER α and T-bet.

Conclusions: Insulin may change the gene expression pattern through T-bet-mediated disruption of master cell-type-specific transcriptional network including GATA-3, ER α , and FOXA1 that dictates the phenotype of hormone-dependent luminal type A breast cancer. T-bet may serve as a marker to differentiate FOXA1+/GATA-3+ breast cancers that may have progressed to hormone-independence.

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P28-2: FUNCTIONAL RELATIONSHIPS BETWEEN HER2 AND THE LEPTIN SYSTEM IN BREAST CANCER

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Background: The obesity hormone leptin (Ob) has been implicated in tumorigenesis, especially in the development of breast cancer (BC). The mitogenic, angiogenic, and antiapoptotic activities of Ob are mediated through the leptin receptor (ObR). Data obtained in HEK293T cells engineered to coexpress ObR and the oncoreceptor HER2 suggested that Ob can transactivate HER2 via ObR. To address this putative interaction, we studied whether simultaneous expression of Ob, ObR, and HER2 can occur in human BC. In cellular models, we studied if ObR and HER2 can physically interact and if activation of Ob/ObR is able to transactivate HER2.

Material and Methods: The expression of Ob and ObR was evaluated by immunohistochemistry in 59 BCs (31 HER2-positive, 28 HER2-negative). Ob and ObR were classified as positive (at least +) or negative (below +). The relationships among Ob and ObR and the clinicopathological features, that is, grading (G1, G2, and G3), tumor size (diameter in mm), node involvement (positive or negative), vascular invasion (positive or negative), and ER and PgR expression (positive or negative) were analyzed using the Chi square test. The mechanistic relationships between ObR and HER2 were studied by western blotting, co-immunoprecipitation, and immunofluorescence/deconvoluted microscopy in MCF-7 cells that are sensitive to Ob and coexpress both receptors.

Results: Ob and ObR were coexpressed in 78% of BC and were correlated in all BCs (p10 mm) node-positive tumors (trends $p=0.06$ and $p=0.08$). The simultaneous expression of Ob/ObR and HER2 was found in 39% of BCs, but the Ob/ObR system was also frequent in HER2-negative BCs. Ob, ObR, and combined Ob/ObR did not correlate with HER2, grading, VI, and ER/PgR. Using MCF-7 cells, we found that a

fraction of ObR and HER2 are colocalized and can be found in one immunocomplex. We also demonstrated that 100 ng/mL Ob can activate HER2 tyrosine phosphorylation upon 15 min–1 h treatment.

Conclusions: Ob and ObR are often coexpressed, and a subset of Ob/ObR-positive tumors exhibits concomitant expression of HER2. In BC cells, HER2 and ObR may physically interact and Ob can cross-activate HER2. Thus, high levels of Ob found in obese patients might lead to the activation of the Ob/ObR/HER2 signaling in BC contributing to the resistance of BC to anti-HER2 treatments.

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P28-3: PROLACTIN PROMOTES MAMMARY TUMORIGENESIS SECONDARY TO LOSS OF p53

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Multiple factors and pathways contribute to mammary tumorigenesis. Two factors that have separately been implicated in breast cancer are the hormone prolactin (PRL) and the tumor suppressor p53. This study has begun to examine the interaction between these factors using two mouse models: p53^{-/-} mice made congenic on the FVB/N background and the NRL-PRL transgenic mouse model which employs a non-hormonally regulated, mammary selective promoter to drive expression of PRL in mammary epithelium. Mammary gland transplants were performed on the following genotypes: wildtype, p53^{-/-}, NRL-PRL/p53^{+/+}, and NRL-PRL/p53^{-/-} to circumvent the problem that p53^{-/-} mice are prone to multiple tumors. This study demonstrates that increased PRL combined with loss of p53 cooperatively affect mammary tumorigenesis in multiple ways. Tumor latency is decreased. Mean survival of the NRL-PRL/p53^{-/-} recipients was 209 days as compared to 247 days for the p53^{-/-} recipients. In addition, the tumors appeared to be more aggressive. For the NRL-PRL/p53^{-/-} tumors, 5/15 (33%) invaded into the peritoneal cavity. In contrast, this was not observed for the p53^{-/-} tumors 0/9 (0%); all were confined to the mammary fat pad. However, NRL-PRL/p53^{-/-} and p53^{-/-} tumors were similar histologically. Both were highly anaplastic and were identified as either carcinosarcomas, spindle cell tumors or adenocarcinomas, indicating that loss of p53 appears to dictate the tumor histology. Thus, it appears that PRL and p53 pathways interact to promote breast cancer by decreasing latency and increasing invasiveness. Further studies are being done to examine the contribution of genomic instability to this process.

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P28-4: INHIBITION OF ESTROGEN-INDUCED GROWTH OF BREAST CANCER CELLS BY MODULATING IN SITU OXIDANT LEVELS

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The purpose of our BCRP-funded proposal (BC051097) was to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, i.e., estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle. We previously reported that 17- β -estradiol (E2)-induced mitochondrial (mt) reactive oxygen species (ROS) act as signaling molecules. Here we have examined whether antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as catalase and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA, by real-time RT-PCR, the rate of DNA synthesis by BrdU incorporation, and different phases of cell cycle by flow cytometry. We also determined the morphology and behaviors of cells that overexpress mtSOD, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Our data revealed that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells that is evident from the lower incorporation of BrdU in siRNA treated cells compared to wild-type cells in the presence of E2. We observed similar results by flow cytometry. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antioxidant treatment and detoxification of ROS prevented E2-induced expression of cyclin D1 and pna, markers of cell proliferation detected by Real time PCR. In cells overexpressed with adenovirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their siRNA, E2 was not able to produce any colony. Both antioxidants ebselen and N-acetylcysteine produced similar effects. It appears that E2-dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling. Since neither antioxidants nor mito-

chondrial biogenesis blocker used in this study are reported to regulate the ER, our findings suggest that E2-induced mtROS modulates G₁ to S transition and some of the early G₁ genes through a nongenomic, ER-independent signaling pathway. These data indicate that E2-induced mtROS are involved in the regulation of early G₁ phase progression and colony formation of breast cancer cells.

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P28-5: PROGESTERONE RECEPTORS (PR) PROTECT BREAST CANCERS FROM KILLING BY TAXANES

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Background: The taxanes (Tx), paclitaxel (Px) and docetaxel (Dx), are among the most effective treatments for advanced breast cancers. While both taxanes kill cancer cells, clinical studies suggest that Dx is more effective than Px in inducing apoptosis and is often effective in Px-resistant cancers. Response to taxanes is influenced by presence of estrogen (ER) and/or progesterone (PR) receptors in tumors. Using ER+ breast cancer cells, we find that expression of PR generates resistance to Px-induced apoptosis.

Objectives: (1) define molecular mechanisms by which ER and PR suppress tumor response to taxanes, (2) to define the mechanisms underlying differences in response to Dx versus Px, and (3) to identify the genes regulated by taxanes in breast cancers.

Methods: To determine possible mechanisms of PR-mediated resistance to taxane-induced apoptosis, expression profiling was performed in ER+ breast cancer cells with inducible PR. Cells either lacking or expressing PR for 48h were treated with vehicle, Px or Dx for 24h. This allowed us to define genes regulated by taxanes in the absence or presence of PR.

Results: The total number of genes regulated by Px and Dx is similar, and many genes are the same, but a subset of genes are uniquely regulated by each Tx. Four classes of genes are regulated by both Tx and further regulated by PR: Class I is upregulated by Tx and downregulated by PR; Class II is upregulated by Tx and further upregulated by PR; Class III is downregulated by Tx and upregulated by PR; and Class IV is downregulated by Tx and further downregulated by PR. Many of the genes oppositely regulated by Tx and PR control proliferation, cellular metabolism, and apoptosis. These include TNF alpha, Annexin I, and Cyclin B1.

Conclusion: Key genes for both Tx modulate apoptosis. PRs regulate multiple genes, some of which overlap with Tx regulated genes. As a result, the presence of PRs modifies the ability of both Px and Dx to regulate gene expression.

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P28-6: IN UTERO EXPOSURE TO CADMIUM AND MAMMARY CANCER RISK IN RATS

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In utero exposure to high levels of estrogen (or estrogen mimickers) alters mammary development in rats and mice and increases their susceptibility to mammary cancer. Cadmium, a heavy metal with a half-life of over 30 years in the mammalian body, potentially binds to and activates the estrogen receptor. Previous studies have shown that in utero exposure to cadmium—at levels present in some human environments—accelerated puberty onset and altered mammary development in rats. In this study we sought to determine whether in utero exposure to low doses of dietary cadmium altered body weight, mammary development, and ultimately mammary cancer risk.

To test whether in utero exposure to low doses of dietary cadmium have significant effects on mammary development and mammary cancer risk, we exposed pregnant rat dams to a diet consisting of 30% fat (similar to a typical human diet) and very low cadmium doses (0.075 or 0.15 mg/kg feed), throughout pregnancy. Control groups received daily injections of vehicle (corn oil) or estrogen (10 µg dissolved in corn oil) on days 19 and 20 of pregnancy. After parturition, all rats were switched to AIN93 laboratory chow and the offspring were cross-fostered. The effects on (1) birth weight, (2) postnatal weight development, (3) puberty onset, (4) mammary development, and (5) DMBA-induced mammary tumorigenesis were investigated.

The higher cadmium dose induced a long-lasting increase in postnatal body weight that was first detected on post-natal day (PND)5 ($p < 0.04$) and accelerated puberty onset as measured by vaginal opening ($p < 0.03$) compared to controls and lower-dose groups. Mammary development was also significantly altered by in utero exposure to cadmium. At PND50 offspring of lower-dose cadmium-exposed rats had a significantly decreased epithelial density ($p < 0.05$) compared to the estrogen-treated group. Differences in allometric growth were measured by comparing fat pad to mammary

epithelial tree length. An average of 71% of the length of the fat pad was filled with the mammary epithelial tree in the lower-dose cadmium group compared to an average of 53% in the higher-dose group ($p < 0.005$), while the vehicle group had an average of 65% and the estrogen group 67%. Proliferation-to-apoptosis ratio—an indication of cellular turnover—was significantly increased in the lower-dose cadmium group ($p < 0.05$ compared to higher-dose and estrogen treated groups) at PND50. These differences in mammary development were strongly correlated to later mammary cancer incidence. Final mammary tumor incidence was lowest in the lower-dose cadmium group (56% of rats developed tumors) and highest in the higher-dose cadmium group (80% tumor incidence) ($p < 0.001$); 73% of the vehicle-treated rats developed mammary tumors.

This study presents initial findings on the effects of in utero exposure to low doses of dietary cadmium on post-natal body weight, mammary development, and breast cancer risk. Follow-up studies based on this work will aid in breast cancer prevention efforts that include dietary guidelines for pregnant women.

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P28-7: EFFECTS OF ESTROGEN ON INTERCELLULAR COHESION

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Estrogen receptor status is mainly associated with breast tumor growth but it is also highly correlated with epithelial cell differentiation and low tumor malignancy. Thus, the absence of the estrogen receptor alpha is strongly linked to increased metastatic potential and poor patient prognosis. Much work has shed light on how tumors overcome their dependence on estrogen as a growth factor by inducing the expression of other growth factors such as EGF, IGF, Heregulin, etc., but we understand far less regarding the mechanisms by which loss of estrogen sensitivity and resistance to estrogen targeting drugs induces tumor progression. Because metastases is the vector that most often leads to cancer-related lethality, this issue is central for reducing mortality from breast cancer. Here we provide a novel link between estrogen signaling and maintenance of the apical contacts that attach a cell to its neighbors at the tight junction. These data suggest that estrogen promotes the integrity of tight junctions and thereby prevents detachment of cells which is one of the key early steps in the progression of metastases. Because Rich1 expression is also found to be low in metastatic tumor cell lines, it is proposed that the levels of Rich1 expression is important for the drug resistance and or the subsequent increase in the metastases of these tumors. This study will directly address these important issues by identifying the requirement of Rich1 in estrogen-mediated inhibition of tumor invasiveness and by determining whether Rich1 expression is sufficient to at least partially reverse tumor invasiveness. This analysis will potentially provide novel markers such as Rich1 that can be used to evaluate metastatic risk of patient-derived tumors. These studies will also identify new avenues for devising drugs that target Rich1-mediated signaling so that clinical protocols can be devised that maintain the effects of anti-estrogen treatment on tumor growth while preventing the metastatic progression of these tumors.

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P28-8: EXPRESSION AND FUNCTION OF ANDROGEN RECEPTOR COACTIVATOR p44 IN BREAST CANCER

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Hormones and their receptors, including estrogen and its receptor, play an important role in the development and progression of ductal carcinoma. In addition to estrogen receptor (ER), the role of androgen, androgen receptor, and its coactivator in tumorigenesis of ductal carcinoma has been recently recognized. p44 is a recently identified androgen receptor coactivator that enhances androgen receptor-mediated transcriptional activity in a ligand-dependent manner. In prostate cancer, p44 is expressed in the nucleus of benign epithelial and translocated into cytoplasm in cancer. Further, its nuclear expression inhibited prostate cancer growth. In this report, we examined the expression and function of p44 in breast cancer. In contrast to findings in prostate cancer, the expression of p44 shows strong cytoplasmic expression in morphologically normal terminal ductal lobular units while nuclear p44 is observed in both ductal carcinoma in situ (DCIS) and invasive carcinoma. Further, overexpression of nuclear-localized p44 stimulates proliferation and invasion in MCF7 breast cancer cells in the presence of estrogen. These findings strongly suggest that p44 plays a role in mediating the effects of hormones during tumorigenesis of ductal carcinoma of breast.

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P28-9: PREGNANCY-MEDIATED BREAST CANCER PROTECTION: DOES EPIGENETIC REGULATION OF GROWTH HORMONE (GH) AXIS AND GENE EXPRESSION IN MAMMARY CELLS PLAY A ROLE?

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During pregnancy, the mammary gland is exposed to the highest physiological concentrations of E+P. Early full-term pregnancy significantly lowers subsequent risk of developing breast cancer in humans, suggesting that hormone exposure alters biology of the breast epithelial cells. These phenomena can be mimicked in rodents, where short-term E+P treatment is equally protective. Although the mechanism of pregnancy protection remains largely unknown, it is most likely to be permanent given that there is a life-long protection in both rodents and humans. Such permanent mechanisms are most probably mediated by epigenetic regulation of gene expression through DNA methylation and covalent histone modifications. We hypothesize that E+P-mediated epigenetic changes occurring at both the systemic (such as GH axis) and local (mammary gland) levels alter specific regulatory pathways, thereby protecting the breast from carcinogenesis.

Sprague-Dawley (SD) and Wistar-Furth (WF) female rats were bred to proven males at 53 days, weaned, and allowed to naturally involute for 28 days. On day 28, both parous and nulliparous females were surgically implanted with a silastic cannula into the right external jugular vein. The next day, parous and aged-matched nulliparous females from both strains were bled from the right external jugular vein every 30 minutes for 4 ½ hours, then serum, pituitaries, and mammary glands were collected to analyze the expression of GH and critical mammary gland genes.

Mean serum GH levels were significantly decreased ($p < 0.01$) in parous versus nulliparous controls in both rat strains. Individual pulse profiles showed that parity significantly ($p < 0.05$) decreased GH pulse amplitude in both rat strains. Furthermore, mean serum GH levels were reduced ($p < 0.05$) during estrus and throughout diestrus ($p < 0.05$) in both parous strains versus nulliparous controls. However, QPCR analysis revealed that there was no significant change in GH mRNA in the pituitaries of parous rats compared to nulliparous rats.

Areg, is a ligand for the EGF-R and plays an important role in mammary epithelial cell proliferation and cancer development. *Sparc* is involved with extracellular matrix formation and *Nnat* is involved in protein transport. *Areg*, *Sparc*, and *Nnat* mRNA expression in the mammary glands was downregulated due to parity. This evidence suggests that parity results in alteration of systemic GH levels and both mammary epithelial and stromal gene expression. Bisulfite genomic sequencing assays to see if these genes are methylated in their promoter CpG islands are currently ongoing.

This study will give helpful insights into understanding how a parity-associated altered systemic hormone milieu and altered gene expression in the mammary gland protect the breast from carcinogenesis.

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P28-10: EVIDENCE THAT FETAL ADRENAL AND PLACENTAL FUNCTION MAY INFLUENCE LIFETIME RISK FOR BREAST CANCER

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Estradiol (E2), the most abundant estrogen late in human pregnancy, is essential for providing nutrients for fetal growth and removing metabolic end products by enhancing uterine blood flow. While early investigators believed that placental estradiol (E2) played this role, they failed to recognize that E2 is largely bound to sex hormone binding globulin and not available to target cells. The important role of E2 in fetal growth has been recognized in numerous clinical studies. Previously, Dr. Siiteri proposed that the E2-stimulated blood flow to the maternal breasts during gestation acts to reduce tissue levels of accumulated mutagens. In support of this novel hypothesis for explaining the protective effect of pregnancy against breast cancer, we previously found a reciprocal relationship between serum E2 levels and the subsequent incidence of breast cancer in mothers in the Child Health and Development Studies (CHDS) pregnancy cohort. Placental E2 is produced from dehydroepiandrosterone sulfate (DHEAS) secreted by the fetal adrenal glands and then hydroxylated at the 16 position in the fetal liver. Therefore, we also investigated if mothers' pregnancy serum E2 levels are related to adult serum DHEAS levels in their daughters. Subjects in this project are CHDS daughters who provided serum samples at ages 27–30 during a study conducted by Dr. J. Richard Udry in 1990–91. Higher E2 levels in maternal pregnancy serum predicted faster intrauterine growth and, independently, higher serum DHEAS levels in adult daughters. Rate of growth in childhood was an additional, independent predictor of adult DHEAS. Adult DHEAS was highest in women

who were both heavy at birth and gained more weight in middle childhood. These women had earlier menarche and higher body mass at adolescence and at ages 27–30. Conversely, adult DHEAS was lowest among women who were lighter at age 9 despite higher birthweight, had later menarche and remained thinner at adolescence and at ages 27–30, and therefore, would probably be at higher risk for premenopausal breast cancer. These findings suggest a role for fetal adrenal and placental function and possibly DHEAS in lifetime risk for breast cancer.

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P28-11: ADIPOSE ESTROGEN AND INCREASED BREAST CANCER RISK IN OBESITY: REGULATION BY LEPTIN AND INSULIN

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The increased risk of breast cancer in obesity is highly correlated with serum estrogen levels. A potential link between obesity and breast cancer risk is estrogen production by the adipose tissue, a major site of post-menopausal estrogen synthesis. Adipose tissues produce aromatase that catalyzes estrogen synthesis from androgen and also 17- β -hydroxysteroid dehydrogenase (17- β HSD) important for the conversion of estrone to estradiol. However, mechanisms regulating adipose expression of these genes in obesity are poorly understood. We hypothesized that hyperinsulinemia and hyperleptinemia associated with obesity contribute to elevated adipose expression of aromatase and 17- β HSD and thereby to increased estrogen production. We used real-time quantitative PCR to initially determine aromatase and 17- β HSD gene expression from total RNA isolated from adipose tissues of two models of murine obesity; the genetically obese ob/ob mice, and a model of diet-induced obesity in C57BL/6J mice where mice were fed a high fat diet (HFD; 60% kcal from fat) or a low fat diet (LFD; 10% kcal from fat) for 16 weeks. On the HFD the mice became obese and expressed elevated plasma insulin and leptin. Aromatase and 17- β HSD mRNA expression was significantly increased in the adipose tissues of mice on the HFD compared to those on the LFD. Interestingly, the expression of these genes was reduced in the leptin-deficient ob/ob mice compared to its lean counterparts. Treatment of either ob/ob or lean mice with exogenous leptin increased adipose expression of aromatase and 17- β HSD mRNA, suggesting that their expression is at least in part mediated by a leptin-dependent pathway. This was further confirmed in vitro using cultured 3T3-L1 adipocytes. While aromatase and 17- β HSD expression decreased during the course of adipocyte differentiation, treatment of mature adipocytes with leptin dramatically increased the expression of these genes. Aromatase mRNA was significantly induced in adipocytes at 3 and 6 hr after leptin treatment while 17- β HSD expression was reduced at 3 hr but significantly increased after exposure to leptin for 24 hr. Thus long-term chronic exposure to leptin may increase the expression of β HSD5 from adipocytes. We next determined the regulation of aromatase and 17- β HSD gene expression in response to insulin. Insulin treatment also induced a dramatic and significant expression of aromatase and β HSD5 gene expression in the adipose tissues of both C57BL/6J lean and in genetically obese ob/ob mice that are metabolically insulin resistant. Mature 3T3-L1 adipocytes in culture also responded to insulin treatment with dramatic increases in aromatase and 17- β HSD5 expression. Both genes were induced significantly as early as 1 hr after insulin exposure and continued to increase up to 3 hr of insulin treatment. Taken together these data suggest that hyperinsulinemia and hyperleptinemia associated with obesity may increase breast cancer risk by inducing the adipose expression of both aromatase and 17- β HSD, molecules important for the production of biologically active estrogen. Studies under way to identify insulin/leptin mediated signaling pathways in the adipocyte that lead to increased aromatase and 17- β HSD synthesis may identify unique targets to attenuate estrogen production from the adipose tissues in obesity.

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P28-12: ESTROGEN DEPRIVATION AND INHIBITION OF BREAST CANCER GROWTH BY ACTIVATING THE NUCLEAR RECEPTOR LXR

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Estrogen plays an important role in normal physiology. It is also a risk factor for breast cancer and anti-estrogen therapies have been shown to be effective in the treatment and prevention of breast cancers. The liver is important for estrogen metabolism and a compromised liver function has been linked to hyperestrogenism in patients. In this report, we showed that the liver X receptor (LXR) controls estrogen homeostasis by regulating the basal and inducible hepatic expression of estrogen sulfotransferase (Est, or Sult1e1), an enzyme critical for metabolic estrogen deactivation. Genetic or pharmacological activation of LXR resulted in Est induction, which in turn inhibited estrogen-dependent uterine epithelial cell proliferation and gene expression, as well as breast cancer growth in a nude mouse model of tumorigenicity. We further established that Est is a transcriptional target of LXR and deletion of the Est gene in mice

abolished the LXR effect on estrogen deprivation. Interestingly, Est regulation by LXR appeared to be liver-specific, further underscoring the role of liver in estrogen metabolism. Activation of LXR failed to induce other major estrogen metabolizing enzymes, suggesting that the LXR effect on estrogen metabolism is Est-specific. In summary, our results have revealed a novel mechanism controlling estrogen homeostasis in vivo and may have implications for drug development in the treatment of breast cancer and other estrogen-related cancerous endocrine disorders.

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P28-13: MAMMARY TUMOR PROGRESSION IN A NOVEL RAT MODEL OF CHILDHOOD ONSET OBESITY

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Obesity is a risk factor for breast cancer incidence and mortality. The epidemic of childhood obesity emphasizes a need to define the relationship between early onset obesity and breast cancer. Toward this, we developed a unique childhood onset Diet Induced Obese (DIO) model of breast cancer that mimics progression of human breast cancer. We exploit this model to delineate the influence of obesity and diet on mammary tumor onset, phenotype, and architecture. In this model of obesity, a "Western Diet" high in fat and simple carbohydrates was used, representing a typical human diet of North America. Briefly, weanling outbred Sprague Dawley rats were randomly placed on either a Western or Rat Chow control diet. After 5 weeks, animals were divided into Western Obese, Western Diet Resistant Lean, and Rat Chow Lean groups based on body fat mass measured by DEXA scan. Western Obese rats had a 2-fold increase in fat mass, compared to each Lean group. No significant differences in net assimilated energy consumption existed among groups, indicating hyperphagia was not responsible for fat mass differences. Western Obese rats had elevated serum comorbidity factors, insulin, leptin, glucose, free fatty acids, and triglycerides compared to Lean Rat Chow Controls. When each group was treated with the carcinogen methylnitrosourea, mammary tumors appeared sooner in Obese and Lean rats on the "Western Diet," compared to Lean Chow rats. The Obese group had the highest tumor numbers, showed a predominantly invasive tumor phenotype via histology and had lower estrogen receptor alpha levels, as compared to each Lean group. Growth curves revealed tumors of Western fed rats initially grew faster, compared to Chow controls. Interestingly, by studies end tumors of Obese rats were significantly smaller compared to Lean Western and Lean Chow groups. These findings are in accord with the association between obesity and breast cancer morbidity in humans. As increasing evidence indicates tissue microenvironment affects tumorigenesis, rat mammary tissues and tumors were analyzed with nonlinear optical imaging technologies. We are the first to use Coherent Anti-Stokes Raman Scattering and Second Harmonic Generation on the same platform to simultaneously image tumor cells, adipocytes, and collagen fibrils, a technique not requiring tissue labeling. Multiphoton imaging of mammary stroma revealed a significant impact of diet and obesity on adipocyte and collagen 3-D structure, parameters not accessible by standard histology. Adipocyte size increased in Obese and Lean rats on a Western Diet, suggesting increased fat mass of Obese rats results from adipocyte proliferation. Obese rats contained increased collagen levels that positively associated with tumor aggressiveness. These studies provide new insights into the relationship between obesity and breast cancer. In summary, our results demonstrate a Western diet at an early age can result in an earlier onset of cancer, regardless of body fat levels. Also, obesity in the context of a Western diet has significant impact on comorbidity factors, mammary progression, and microenvironment architecture. Our approach using a unique animal model of obesity and novel imaging technologies represents a valuable system to better understand adipocyte-epithelial interactions during tumorigenesis, establish biomarkers for cancer prevention and prognosis, and to identify new therapeutic targets for aggressive obesity-associated cancers.

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P28-14: DYSREGULATION OF Zip6 (LIV1) IS POTENTIATED BY PROLACTIN IN TUMORIGENIC BREAST CELLS

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Breast cancer is one of the leading causes of death among women. The etiology is unknown; however, compelling evidence implicates dysregulated zinc (Zn) homeostasis in breast cells. Tight regulation of cellular Zn metabolism controls programmed cell death (apoptosis), a process which is uncoupled in cancer. We speculate this is due to dysregulation of Zn transporting mechanisms in breast cells. Zip6 (LIV-1) transports Zn into the cell. Studies in humans have suggested that Zip6 gene expres-

sion may be a useful prognostic marker for breast cancer. However, the functional relevance of altered Zip6 expression is not understood.

We hypothesize that alterations in Zip6 dysregulates intracellular Zn pools affecting the ability of cells to undergo apoptosis. To begin to test this hypothesis, we measured Zip6 gene expression in cultured tumorigenic (T47D) and normal (HME) human breast cells using quantitative real-time PCR and determined that Zip6 expression was 2-fold lower in tumorigenic cells, similar to what has been reported from breast biopsies. Paradoxically, Zip6 protein abundance was ~10-fold higher. Moreover, in normal breast cells, Zip6 resides almost exclusively on the plasma membrane reflecting its normal role as a Zn import protein. In contrast, ~90% of Zip6 is located intracellularly in tumorigenic breast cells. Confocal microscopy indicated that intracellular Zip6 is restricted to a unique, yet unknown compartment. The identification of this compartment and its role in cellular Zn homeostasis is of critical interest as abnormal protein localization is often linked to metabolic cell disorders. Taken together, this suggests that over-expression and mislocalization of Zip6 protein may play a role in the dysregulation of cellular Zn metabolism and thus contribute to breast cancer pathogenesis.

Recently, a relationship between elevated prolactin levels and breast cancer has been observed. Clinical implications reflect the fact that drugs that are widely prescribed for treatment of a range of mental and neurodegenerative illnesses including Parkinson's and depression (e.g., dopamine antagonists) secondarily increase prolactin levels, potentially increasing breast cancer risk in an extraordinary number of individuals. Understanding the relationship between prolactin and breast cancer is of critical importance and may result in novel therapies. We previously determined that prolactin affects Zn metabolism in normal breast cells. Understanding the mechanisms responsible for this regulation and its potential dysregulation in disease is our long-term goal. Thus, we postulate that prolactin plays a regulatory role in Zip6 expression and function. To begin to test this hypothesis, we treated tumorigenic breast cells with prolactin and determined that prolactin significantly decreased Zip6 mRNA level (2-fold) while Zip6 protein abundance was concomitantly increased. This suggests that exposure to prolactin may affect breast cell Zn metabolism through further dysregulation of Zip6. We are currently exploring the effects of prolactin in normal breast cells and the functional consequences. In summary, we have begun to examine the role and regulation of Zip6 in tumorigenic breast cells to provide an understanding of its relevance as a potential prognostic marker for breast cancer. Elucidating novel prognostic markers and understanding their role in breast cancer initiation and progression is critical to developing efficacious therapies.

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P28-15: THE INDUCIBLE 6-PHOSPHOFRUCTO-2-KINASE (PFKFB3) TRAFFICS TO THE NUCLEUS AND STIMULATES CELL PROLIFERATION

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Phosphofructo-1-kinase, a rate-limiting enzyme of glycolysis, is activated by fructose-2,6-bisphosphate, a product of four 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (isoforms PFKFB1-4). The inducible PFKFB3 isoform has been found to be increased in neoplastic cells and required for the anchorage-independent growth of *ras*-transformed cells. We examined the relative PFKFB3 protein expression within the cytoplasmic and nuclear fractions of MCF-7 and HeLa cells and surprisingly found that PFKFB3 is predominantly expressed in the nucleus rather than the anticipated cytoplasm. We then identified through computational modeling a nuclear localization domain and using site-directed mutagenesis, found that two lysines within this domain (K472, K473) are required for the routing of ectopically expressed PFKFB3 to the nucleus. Several nuclear proteins are required for the regulation of growth and we thus investigated the possible role of nuclear PFKFB3 in cell proliferation. We transfected cells with plasmids containing wild-type (i.e., nuclear) or re-routed cytoplasmic PFKFB3 (mutant K472/473A) and examined the effects on cellular proliferation. Whereas expression of the wild-type nuclear PFKFB3 stimulated a 50% increase in cell proliferation, the K472/473A cytoplasmic mutant had no effect (vector, $1.2 \pm 0.026 \times 10^6$ cells/well; wild-type, $1.89 \pm 0.011 \times 10^6$ cells/wells; K472/473A mutant, $1.42 \pm 0.016 \times 10^6$ cells/well; $p=0.0006$). We also determined that overexpression of a kinase-dead mutant of PFKFB3 in cells failed to stimulate cell proliferation. Last, we found that the expression of PFKFB3 in the nucleus relative to the cytoplasm is increased during the S and G2/M phases of the cell cycle. In conclusion, these findings demonstrate that the localization of PFKFB3 to the nucleus during the S and G2/M phases of the cell cycle functions in part to stimulate the proliferation of transformed cells.

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P28-16: DEFECTIVE ENERGY METABOLISM AND REGULATION OF NUCLEAR GENE EXPRESSION BY CANCEROUS MITOCHONDRIA IN BREAST CANCER TRANSMITOCHONDRIAL CYBRIDS: A NOVEL APPROACH

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Background: Somatic mitochondrial DNA (mtDNA) mutation in cancer cells is a common phenomenon. However the functional effect of altered mitochondria on cancer development has never been established. To understand the role of cancerous mitochondria, the effect of the altered nuclear genome must be separated. Alteration in energy metabolism is a hallmark of cancer. In cancer cells mitochondrial oxidative phosphorylation is down-regulated while glycolytic enzymes are up-regulated. Using transmitochondrial cybrid cells, the functional effect of cancerous mitochondria can be studied and compared to mitochondria derived from normal breast epithelial cells in a defined nuclear background. Altered mitochondrial function including defective oxidative phosphorylation can be demonstrated. Furthermore, since mitochondrial biogenesis and function depend mainly on the expression of nuclear encoded proteins, we hypothesize that altered mitochondria from cancer cells could regulate nuclear gene expression through crosstalk mechanism.

Methods: Transmitochondrial cybrid cell lines were established by fusing the mitochondria-depleted rho zero cells derived from osteosarcoma cell line 143B.TK- with mitochondria derived from enucleated tumorigenic (MDA-MB-231 and MDA-MB-468), non-tumorigenic (MDA-MB-436 and MDA-MB-453) breast cancer cell lines, or normal breast epithelial cell line (MCF-10A). Cell viability and mitochondrial functions including oxygen consumption, respiratory chain enzyme activities, and ATP synthesis were studied under conditions of metabolic stress. The total RNA were isolated from the cybrid cell lines, and gene expression profile was studied using Affymetrix Human Genome U133 plus 2.0 genechip.

Results: Defective mitochondrial function was demonstrated in cybrids containing cancerous but not normal mitochondria. One of the molecular etiologies of the defect was identified in at least one breast cancer cell line, MDA-MB468. The mutation was 16021C>T in the amino acid acceptor stem region of mitochondrial tRNA proline gene, which affects overall mitochondrial protein synthesis. Principal Component Analysis (PCA) of the gene expression profile revealed distinctive sample segregation between the groups carrying mitochondria derived from tumor and normal cell. The segregation within the tumor group also correlated with tumorigenic potential of the derived mitochondria. Subsequent analyses of commonly enriched functional-related gene groups in breast cancer group demonstrated a subset of differentially expressed genes involved in mitochondria-mediated apoptosis, energy, and fatty acid metabolic processes.

Conclusion: For the first time the functional effect of mitochondria derived from breast cancer cells was studied in a defined nuclear background. Our results suggest that cancer cells harbor altered mitochondria that can talk back to the nucleus and actively participate in the regulation of gene expression in breast cancer development.

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P28-17: PROLACTIN ACTIVATION OF Jak1 POSITIVELY MODULATES PROLACTIN-Jak2 SIGNALING IN BREAST CANCER CELLS

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Prolactin receptors (PRLR) have been considered selective activators of tyrosine kinase Jak2 but not Jak1, Jak3 or Tyk2. Prolactin (PRL) signaling through the Jak2-Stat5 pathway has been well characterized and implicated for the normal growth, development, and differentiation of human breast epithelia. PRL has also been suggested to have a breast tumor-promoting effect in humans, and more than 95% of breast cancers express PRLR. We now report marked PRL-induced tyrosine phosphorylation of Jak1, in addition to Jak2, in a series of human breast cancer cell lines, including T47D, MCF7, and SKBR3. In contrast, PRL did not activate Jak1 in immortalized, non-cancerous breast epithelial lines HC11, MCF10A, ME16C, and HBL-100, or in CWR22Rv1 prostate cancer cells or MDA-MB-231 breast cancer cells. Introduction of exogenous PRLR into MCF10A, ME16C, or MDA-MB-231 cells reconstituted both PRL-Jak1 and PRL-Jak2 signals. PRL stimulated the enzymatic activity of Jak1 in T47D cells, as verified by *in vitro* kinase assays, and PRL activated Jak1 and Jak2 with indistinguishable time and dose-kinetics. Relative Jak2 deficiency did not cause PRLR activation of Jak1 since overexpression of Jak2 did not interfere with PRL activation of Jak1. Instead, PRL activated Jak1 through a Jak2-dependent mechanism, based on disruption of PRL activation of Jak1 following Jak2 suppression by (1) lentiviral delivery of Jak2 shRNA, (2) adenoviral delivery of

dominant-negative Jak2, and (3) AG490 pharmacological inhibition. Finally, suppression of Jak1 by lentiviral delivery of Jak1 shRNA blocked PRL activation of ERK and Stat3 and suppressed PRL activation of Jak2, Stat5a, Stat5b, and Akt, as well as tyrosine phosphorylation of PRLR. The data suggest that PRL activation of Jak1 represents a novel, Jak2-dependent mechanism that may serve as a regulatory switch leading to PRL activation of ERK and Stat3 pathways while also serving to enhance PRL-induced Stat5a/b and Akt signaling. From a pharmacological perspective, the novel involvement of Jak1 in PRLR signaling, at least in a subset of breast cancer, may represent a new pharmacological target. Specifically, combined inhibition of Jak2 and Jak1 may synergize to suppress growth and survival-promoting PRL effects in some tumors and be advantageous over inhibition of Jak2 alone. Furthermore, if Jak1-specific pathways were to preferentially mediate tumor-promoting effects of PRL, inhibitors of Jak1 may be useful in breast cancer treatment to preferentially disrupt select PRL-induced signals while having less effect on other signaling pathways. Ongoing work aims to determine the role of Jak1 in PRL biology and signaling in breast cancer and to further investigate the molecular mechanisms underlying PRL-Jak2 activation of Jak1.

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P28-18: TRANSCRIPTIONAL REPRESSION OF ER α BY HEXIM1 AND ITS ROLE IN MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

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Estrogens contribute to tumorigenic growth in the mammary gland but the precise mechanisms are not well understood. Our laboratory has reported that 17- β estradiol (E2) decreases the expression of HEXIM1, a breast cell growth inhibitor. We now report that HEXIM1 inhibits estrogen receptor- α (ER- α) transcription via inhibition of the Positive transcription elongation factor b (P-TEFb). During the elongation phase of transcription, P-TEFb hyperphosphorylates RNA Polymerase II (RNAP II) and this is a hallmark for productive messenger RNA (mRNA) generation. Using chromatin immunoprecipitation assays, we found that a transient increase in HEXIM1 expression in MCF-7 human breast cancer cells led to a decrease in E2-induced recruitment of P-TEFb and hyperphosphorylated RNAP II to the 3' region of the ER- α target genes, pS2 and Cyclin D1. The recruitment of hypophosphorylated RNAP II remains unchanged at similar regions of pS2 and Cyclin D1. Conversely, decreasing HEXIM1 expression in MCF-7 cells by siRNA relieves repression of HEXIM1 on P-TEFb recruitment to pS2. This indicates that HEXIM1 inhibits the elongation phase of ER- α -mediated transcription. To investigate a functional role for ER- α /P-TEFb/HEXIM1 interactions, we developed MMTV-HEXIM1 transgenic mice with inducible expression of HEXIM1 in the mammary gland. Adult mice at 9 weeks were ovariectomized and treated with E2. Immunohistochemical staining showed that an increase in HEXIM1 expression inhibits ductal branching due to a decrease in proliferation and an increase in apoptosis. This correlates with a decrease in Cyclin D1 and hyperphosphorylated RNAP II expression quantified by Western blot analyses and immunohistochemistry. The C-terminus of HEXIM1 is necessary for its interaction with ER- α and P-TEFb and also for its inhibitory function, so we developed a knock-in mutant mouse model that expresses HEXIM1 with a partial C-terminus disruption. We found that these mice have a higher incidence of developing carcinogen-induced mammary tumors when compared to their wild-type littermates. These findings suggest a novel mechanism for the regulation of ER- α transcription via HEXIM1 both *in vitro* and *in vivo*, making HEXIM1 an attractive target for breast cancer therapeutics.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0426.

P28-19: THE ROLE OF Sp1 AND Sp3 IN THE REGULATION OF TFF1 GENE IN MCF-7 CELLS

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To understand how Sp1 and Sp3 involve in the estrogen-regulated TFF1 (pS2) gene transcription, chromatin immunoprecipitation (ChIP) was used to examine the association of estrogen receptor α (ER), Sp1 and Sp3 with the endogenous TFF1 gene promoter in MCF-7 (T5) cells. ER was found to associate with TFF1 gene rapidly after estrogen treatment. Both Sp1 and Sp3 associate with TFF1 gene promoter before and after estrogen treatment although at different levels. Using reChIP, we demonstrated that Sp1 and Sp3 do not associate with the same TFF1 promoter. The co-occupancy of ER and Sp1 on TFF1 promoter remains at a similar level with and without estrogen while that of ER and Sp3 increased in the presence of estrogen. Furthermore, we observed increased co-occupancy of Sp3 and CTD domain serine 5 phosphorylated RNA Polymerase II, the transcription initiating form, on TFF1 promoter after estrogen treated the cells for 45 minutes. Additionally, increased lysine acetylation and sumoylation were found in presence of estrogen and more Sp3 co-occupy the TFF1 promoter together with sumoylation. Taken together, Sp3, instead of Sp1, is

preferentially associated with the estrogen-activated TFF1 promoter. The balance between lysine acetylation and sumoylation of Sp3 or other proteins could be one way to regulate the estrogen-induced gene transcription.

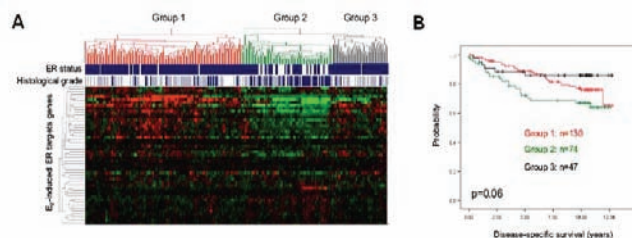
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0284.

P28-20: MECHANISMS OF GENE ACTIVATION AND GENE REPRESSION DETERMINE BREAST CANCER PROGNOSIS

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It is well established that estrogen is linked to breast cancer etiology. However, the molecular mechanisms of estrogen receptor (ERa)-mediated transcriptional activity at a genome-wide level are just beginning to be explored. We utilized genome-wide chromatin immunoprecipitation experiments to identify the genes that recruit ERa in MCF-7 cells when stimulated with estrogen (Kwon et al., 2007). Microarray expression analysis was performed to characterize the estrogen-regulated transcriptome. Interestingly, many genes that recruit ERa appear not to be regulated by it in MCF-7 cells. However, some of them are indeed induced in another cell line (U2OS), indicating that transcriptional cofactors (such as histone modifications) play an important role in determining the cell-specific functions of ERa (Garcia-Bassets et al., 2007). Focusing on the set of genes that is induced by estrogen and shows ERa recruitment in MCF-7 cells, we used a comprehensive set of expression data from 251 breast cancer patients for unsupervised hierarchical clustering. We found a direct correlation between gene expression and patient histological grade (Figure 1a). Many genes were strongly suppressed in cluster group 2, which displays an advanced tumor grade, is ERa negative, and is associated with a patient group that has a reduced survival rate as compared to the two other groups (Figure 1b). The work illustrates a general strategy for disease etiology studies by combining expression profiling with location analysis of key transcriptional regulators. The results also emphasize the importance of gene repression. Indeed, microRNAs have been increasingly implicated in cancer development, and we have begun to examine the role of microRNAs in determining gene repression programs. Interestingly, our analysis suggests that similar to gene expression activators, microRNAs function as gene repressor factors whose cell type-specific activity is determined by additional cofactors.



Estrogen-induced gene expression and the biological relevance of direct ER target genes

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This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0277; National Cancer Institute, Vitamin Cases Consumer Settlement Fund; and National Human Genome Research Institute.

P28-21: AUTOREGULATION OF ESTROGEN RECEPTOR-ALPHA EXPRESSION IN BREAST CANCER CELLS

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Estrogen receptor-alpha (ER) is a key prognostic and therapeutic target for breast cancer patients. The response of breast tumors to endocrine therapy is greatly dependent on the expression of ER in the cells. ER is a member of the nuclear receptor superfamily of transcription factors and mediates the physiological effects of estrogen in cells by activating or repressing its target genes. The expression of ER itself is repressed by estrogen, creating a negative feedback loop to control the magnitude of the estrogen response. It has been previously shown that estrogen treatment causes a decrease in ER mRNA, but the mechanism governing this loss remains unknown. This is made more difficult by the complexity of the ER gene (ESR1) that can be transcribed from seven promoters regulated in different manners.

We used the ER-positive breast cancer cell line, MCF-7, as a model to study how estrogen causes transcriptional repression of ER mRNA. Estrogen treatment of MCF-7 cells resulted in a decrease of ER mRNA as measured by quantitative RT-PCR. ChIP assays showed recruitment of ER with estrogen treatment to the proximal A promoter of ESR1 and another recently reported site near the distal E2 promoter but no recruitment to the C or D promoters. Further experimentation with another ER ligand, 4-hydroxytamoxifen (OHT), and breast cancer cell line, T47D, showed a correlation between transcriptional repression of ER and continuous recruitment of ER to the A promoter, but not the region near the E2 promoter. The chromatin state of ESR1 was examined for changes related to transcriptional repression and decreases in acetylated histone H3 levels were observed after 24 hours of estrogen treatment near the A promoter but not at the E2 promoter. Since changes in acetylated histones were observed, histone deacetylase (HDAC) inhibitors were tested for an effect on repression, but repression was insensitive to HDAC inhibition. These findings suggest that ER recruitment to the A promoter of ESR1 is necessary to achieve transcriptional repression, and repression occurs through an HDAC-independent mechanism.

The elucidation of the mechanism by which estrogen controls the level of ER mRNA is an important goal. Studies have shown that increased levels of ER expression are associated with breast cancer risk and development. It is possible that some tumors may lose the ability to downregulate ER expression, causing high levels of ER and subsequent negative consequences. New therapeutics targeting the critical elements identified in this study may help to restore regulation of ER levels and control the growth of their cancer.

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APOPTOSIS

Poster Session P29

P29-1: ERBB4 INTRACELLULAR DOMAIN (4ICD), A NOVEL BH3-ONLY PROTEIN AND ESTROGEN RECEPTOR COACTIVATOR REGULATES TAMOXIFEN-INDUCED APOPTOSIS OF BREAST TUMOR CELLS

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ERBB4 is a unique member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. In contrast to the other three members of the EGFR family (i.e., EGFR, ERBB2, and ERBB3), which are associated with aggressive forms of human cancers, ERBB4 expression seems to be selectively lost in tumors with aggressive phenotypes. Consistent with this observation, we have shown that ERBB4 induces apoptosis when reintroduced into breast cancer cell lines or when endogenous ERBB4 is activated by a ligand. Upon ligand activation, membrane ERBB4 undergoes proteolytic processing to release the ERBB4 intracellular domain (4ICD). We show that cleavage of endogenous ERBB4 is critical for ERBB4 apoptotic activity and results in mitochondrial accumulation of the 4ICD followed by cytochrome C efflux and activation of cellular caspases. We further show that 4ICD harbors a functional BH3 domain and ERBB4 functions as a unique proapoptotic member of BCL-2 family. Clinically, cytosolic but not membrane ERBB4/4ICD expression in primary human breast tumors was associated with tumor apoptosis, providing a mechanistic explanation for the loss of ERBB4 expression during tumor progression. Alternatively, we have shown that ERBB4/4ICD is a transcriptional coactivator and 4ICD directly interacts with estrogen receptor (ER) to selectively potentiate estrogen-regulated gene expression. Furthermore ERBB4 itself is an estrogen-regulated gene and 4ICD contributes to estrogen-induced proliferation of ER positive breast cancer cells. These observations are corroborated clinically where ERBB4 is significantly associated with ER expression in primary breast tumors. Strikingly, the vast majority of patients with tumors coexpressing ERBB4 and ER respond to endocrine therapy whereas 40% of patients expressing ER alone succumb to their disease. Based upon these clinical results, we tested the hypothesis that ERBB4 regulates tumor response to endocrine therapy. In support of this hypothesis, we found that in three independent preclinical cell models, tamoxifen resistance was associated with the suppression of ERBB4 expression. Furthermore, reintroduction of ERBB4 in a tamoxifen-resistant cell line reestablished tamoxifen sensitivity. Tamoxifen treatment of breast cancer cells resulted in translocation of the 4ICD BH3-only protein to the mitochondria and activation of apoptosis. We show that tamoxifen disrupts an estrogen-driven interaction between ER and 4ICD while promoting mitochondrial accumulation of the 4ICD BH3-only protein. Additionally, BCL-2 inhibition of tamoxifen-induced apoptosis and tamoxifen activation of BAK independent of BAX, a unique feature of the 4ICD BH3-only protein, further supports a role for 4ICD during tamoxifen-induced apoptosis. We propose that ERBB4 represents an important tumor marker predicting therapeutic response of breast cancer patients undergoing endocrine therapy.

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P29-2: A NOVEL APPROACH TO REVERSE RESISTANCE TO THERAPY OF APOPTOSIS-DEFECTIVE BREAST CANCER CELLS

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Defects in apoptotic capability that evolve during tumorigenesis promote tumor growth, provide survival advantage, and confound treatment. Recent studies involving apoptosis-defective cells suggest that such cells utilize alternative death mechanisms when faced with stress. From a therapeutic point of view, a cell death response to cytotoxic therapy either by apoptosis, autophagy, or even necrosis, is a productive response to treatment. Yet, many apoptosis-defective tumor cells survive cytotoxic treatments and resume cell proliferation. Autophagy has been observed in cancer cells faced with a variety of metabolic and therapeutic stresses. Autophagy is a highly regulated process during which cytoplasmic materials and damaged organelles are enclosed in double-membrane bound vesicles that are then targeted to lysosomes for degradation. Persistent autophagy that depletes cells of organelles and critical proteins can lead to demise of the cells. However, in multiple apoptosis-defective tumor cell lines, an autophagic response to cytotoxic drugs allowed the tumor cells to survive. We hypothesize that RNAi knockdown of essential Atg proteins in certain apoptosis-defective cells blocks the execution of a protective autophagic response to stress and thus diminishes the ability of the cells to survive. In the absence of an essential Atg protein, the stressed cells will succumb to death by either apoptosis or necrosis, depending on the nature of the apoptosis-defect and the specific cytotoxic drug utilized. To test this hypothesis, we proposed the following objectives: (1) Investigate the development of a protective autophagic response in apoptosis-defective breast cancer cells treated with breast cancer-relevant cytotoxic treatments. (2) Investigate the ability of the RNAi knockdown of essential autophagy proteins to shift the protective autophagic response to cell death by either apoptosis or necrosis. Our preliminary studies investigated the involvement of cytoprotective autophagy in response to TRAIL in colon carcinoma cells with distinctive defects in the TRAIL-

apoptotic cascade. Our studies suggest that various apoptosis defects that block TRAIL-mediated cell death at different points along the apoptotic signaling pathway shift the signaling cascade from default apoptosis toward cytoprotective autophagy. We also obtained evidence that inhibition of such a TRAIL-mediated autophagic response initiates an effective mitochondrial apoptotic response that is caspase-8 dependent. Our studies demonstrate the existence of a double switch between autophagy and apoptosis that can be exploited for TRAIL therapy: on one hand, inhibition of apoptosis by an array of apoptosis defects can invoke protective autophagy in response to TRAIL, representing a switch from default apoptosis to protective autophagy; on the other hand, prevention of such protective autophagy induces apoptotic cell death, representing a second switch from autophagy back to apoptosis. The possibility of potentially reversing multiple mechanisms of TRAIL resistance by the inhibition of autophagy represents a novel concept for the development of a new approach for TRAIL therapy. Currently, we are investigating the involvement of protective autophagy in response to TRAIL of apoptosis-deficient breast carcinoma cells with a focus on the molecular mechanisms that link disabled autophagy to apoptosis.

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P29-3: DOES PLASMA MEMBRANE CALCIUM-ATPASE 2 OVEREXPRESSION PROTECT BREAST CANCER CELLS FROM APOPTOSIS?

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The plasma membrane calcium-ATPase isoform 2 (PMCA2) is highly expressed on the apical membrane of mammary epithelial cells during lactation and is the predominant pump responsible for calcium transport into milk. The lack of PMCA2 causes a low epithelial content in pregnant deafwaddler (dfw-2J) mouse mammary glands due to widespread apoptosis. The apoptosis is likely a result of elevated cytosolic calcium levels due to an imbalance of calcium influx and efflux, suggesting that PMCA2 is important not only for calcium transport into milk but also for maintaining cytosolic calcium levels. Using the Oncomine database and data mining tools, we found that PMCA2 mRNA expression correlates with tumor grade, metastases, estrogen-receptor negativity, docetaxol resistance, and poor 5-year survival in human breast cancer. Likewise, our own tissue microarray studies revealed associations between decreased 20-year survival and high PMCA2 protein levels. We hypothesize that overexpression of PMCA2 and subsequent enhanced intracellular calcium clearance is a mechanism by which breast cancer cells escape apoptosis. The aims of this research are to determine whether PMCA2 expression correlates with cytosolic calcium levels and sensitivity to apoptosis in human breast cancer cell lines and whether the absence of PMCA2 alters tumorigenesis or induces drug resistance in a transgenic model of breast cancer. We surveyed a panel of breast cancer cell lines by QRT-PCR and picked two with divergent PMCA2 expression levels. MDA-MB-231 cells had 400-fold higher PMCA2 expression than T47-D cells. Apoptosis, indicated by cytoplasmic mono- and oligo-nucleosomes, was higher in T47-D cells than in MDA-MB-231 cells. Furthermore, T47-D cells were more sensitive to Taxol-induced apoptosis. These results are consistent with our hypothesis that high levels of PMCA2 allow breast cancer cells to escape apoptosis. Studies to manipulate PMCA2 levels independently within each cell line are currently under way. Intracellular calcium and apoptosis will be measured in MDA-MB-231 cells with RNAi-mediated knockdown of PMCA2 expression and in T47-D cells engineered to overexpress PMCA2. To determine whether loss of PMCA2 alters tumorigenesis or apoptosis in vivo, we are breeding MMTV-neu mice with dfw-2J mice, which lack PMCA2. We plan to measure tumor latency, size, occurrence of metastases, and tumor response to Taxol in MMTV-neu/dfw-2J mice. This work will contribute to our understanding of how breast cancer cells escape apoptosis, an important event in tumor progression and the development of resistance to chemotherapy.

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P29-4: SUPPRESSION OF APOPTOSIS TO IMPROVE BREAST CANCER THERAPY

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Induction of apoptosis is usually considered a desirable goal in breast cancer therapy. Apoptosis, however, is a physiological anticarcinogenic program of normal cells, and tumor cells commonly develop resistance to apoptosis in the course of neoplastic transformation. Apoptosis is a principal form of death in normal cells, and inhibition of apoptosis in mice improves their survival of radiation and chemotherapy. Inhibition of apoptosis in tumor cells often does not increase clonogenic survival of chemotherapy or radiation in vitro; apoptosis-resistant cells merely die by other means or undergo senescence. Furthermore, apoptosis evolved as a noninflammatory form of cell death whereas necrosis promotes inflammation in vivo, which is likely to be beneficial for the destruction of the tumor. We hypothesize that inhibition of apoptosis in a human breast cancer model will improve tumor response to chemotherapy in

vivo by promoting necrotic cell death and local inflammation. Small-molecule inhibitors of apoptosis are being developed although primarily for non-cancer applications. If our hypothesis is correct, such inhibitors should be useful supplements to cancer therapy since they will not only protect the patient from side effects of therapy but may also augment tumor eradication by promoting pro-inflammatory modes of cell death. To test our hypothesis, we are developing a regulated system of apoptosis suppression in a breast cancer cell line. Specifically, we are generating a subline of MDA-MB-231 cells with regulated expression of short hairpin RNA (shRNA) that inhibits BAX, a positive regulator of apoptosis in this cell line. By inhibiting the expression of a proapoptotic gene rather than overexpressing an antiapoptotic gene, we avoid problems associated with supraphysiological protein levels and also better mimic the effect of small-molecule inhibitors. Once the cell line is developed, we will verify that shRNA induction in this cell line inhibits BAX expression and reduces apoptosis upon exposure to Taxol®. We will then determine if the inhibition of apoptosis increases necrosis in vitro after Taxol treatment. For this purpose, we will use time-lapse phase contrast video microscopy to score and quantitate different types of Taxol-induced cell death in MDA-MB-231 cells with or without BAX knockdown. We expect that BAX knockdown will decrease the number of cells dying through apoptosis but increase the number dying through necrosis and that BAX knockdown will have only a small effect on cellular response to different doses of Taxol in colony-formation assays. We will next determine how the inhibition of apoptosis affects in vivo response to Taxol in a xenograft model. For this purpose, MDA-MB-231 cells will be grown as an orthotopic xenograft in nude mice. Mice will be treated with Taxol, with or without Bax knockdown, or untreated, and tumor response to Taxol will be determined by measuring tumor volume over time. Histological analysis of tumor sections will be used to score for markers of apoptosis, necrotic morphology, and the presence of large numbers of infiltrating lymphocytes (a marker of inflammation). This analysis will show how inhibition of apoptosis affects in vivo response to Taxol and whether it is associated with increased necrosis and inflammation in the treated tumors. If inhibition of apoptosis improves tumor response to therapy, this will warrant the use of apoptosis inhibitors in breast cancer treatment.

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P29-5: INDUCTION OF LOW MW PRO-APOPTOTIC FORMS OF THE BCL2 FAMILY MEMBER MCL1 IN BREAST CANCER

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A better understanding of the pathways that control breast cancer cell survival might provide targets for therapy. However, the role of the BCL2 family in this disease is as-yet poorly understood. We monitored the expression of MCL1, a critical member of this family, in a variety of tumor-derived and nontumorigenic breast cell lines. Full-length MCL1L (40-42 KD), which promotes cell viability, was expressed in both types of cells. Unexpectedly, low MW forms of MCL1 (~30-35 KD) were not as prominent in breast cancer as in nontumorigenic cells, where low MW splice variants (e.g., MCL1s/deltaTM) contain only the BH3 region and thus promote cell death. Interestingly, treatment of MCF7 cells with the chemotherapeutic agent etoposide (but not with the growth factor insulin) resulted in a decrease in MCL1L but not in the low MW forms. Furthermore, treatment with EGF followed by beta-estradiol resulted in a switch from predominance of MCL1L to predominance of the low MW forms.

Finally, antisense oligonucleotides were identified that target alternate splicing to MCL1s/deltaTM. These findings suggest that pharmacologic and molecular agents

can be used to induce alternate splicing of antiapoptotic MCL1 to pro-apoptotic forms. This represents a potential novel approach for the treatment of breast cancer.

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P29-6: THE APOPTOSOME AS A THERAPEUTIC TARGET IN BREAST AND BRAIN TUMORS

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Most chemotherapeutic agents trigger cellular damage that results in apoptotic cell death. Typically, this results from release of the respiratory chain protein, cytochrome C, from the intermembrane space of the mitochondria to the cytoplasm. Once cytoplasmic, cytochrome C binds to Apaf-1 protein, which oligomerizes to recruit and activate caspase 9 (a cell death protease) in a structure known as the apoptosome. In many cancers (ovarian, skin, lung, leukemias), it has been demonstrated that chemoresistance can occur as a result of apoptosome suppression in the tumors. This may result either from loss of an apoptosomal component, from post-translational modification of apoptosomal proteins, or from binding of inhibitors to the apoptosome. However, in analyzing a broad panel of tumor types, we were surprised to find that the apoptosome is not inhibited in breast cancers or brain tumors. Rather, the apoptosome was hyperactive in these tumor types, resulting in robust cytochrome C-induced activation of caspase 9 at cytochrome C concentrations that did not promote apoptosome activation in normal cells. These findings raised the intriguing possibility that apoptosome activators might serve to selectively kill breast/brain tumor cells without harming the surrounding normal tissue. Given the similar response of breast and brain tumors to cytochrome C, we wished to determine whether the underlying mechanism responsible for apoptosome activation was similar in these different tumor types. In analyzing malignant breast cells, we found that the complement of apoptosome components was indistinguishable from that found in normal mammary epithelial cells and none of the components appeared to be post-translationally modified. However, we noted that breast cancer cells overexpressed (at both the mRNA and protein levels) a previously characterized apoptosome activator, PHAPI. Through RNAi ablation studies, we were able to demonstrate that reduction of PHAP levels in breast cancer cells eliminated apoptosome hypersensitivity in these cells. Moreover, overexpression of PHAPI in normal breast epithelial cells conferred apoptosome hypersensitivity on these cells. In seeking to determine whether breast and brain tumor cells shared the ability to sensitize the apoptosome through up-regulation of PHAPI, we found that brain tumor cells did not exhibit elevated PHAPI levels. However, unlike breast tumor cells, brain tumor cells appeared to contain elevated levels of Apaf-1. Intriguingly, in several mouse models of brain tumor Apaf-1 appeared to be elevated in the tumors, but entirely absent from the normal brain tissue surrounding the tumors. Thus, unlike in breast tumors, brain tumors appear to modulate cytochrome C sensitivity by altering the expression of a core apoptosomal component. In both breast and brain tumors, the hypersensitivity to cytochrome C led us to look for therapeutic agents that might exploit this sensitivity and promote apoptosome activation. We have begun a screen for cytochrome C-mimetic compounds that can activate the apoptosome in purified breast cancer cell cytosol lacking mitochondria. We have screened several thousand compounds. Although we have isolated several cell-killing compounds, none of the currently isolated compounds appears to kill in an Apaf-1-dependent manner (which is critical for the differential sensitivity of the normal and tumor cells). This screening is ongoing. We have also begun design of cell permeable cytochrome C derivatives that might activate the apoptosome directly in these cells. Our progress in these areas will be discussed.

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P29-7: TREATMENT WITH A BI-SPECIFIC ANTISENSE BCL-2 AND BCL-XL OLIGONUCLEOTIDE, BUT NOT TAMOXIFEN, INDUCES PROGRAMMED CELL DEATH IN A DUCTAL CARCINOMA IN SITU PRIMARY TISSUE EXPLANT

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Tamoxifen is currently used for the prevention and treatment of breast cancer and, although beneficial, has known limitations and secondary risks requiring investigation into novel therapeutic approaches. It has been shown that many premalignant breast lesions, including ductal carcinoma in situ (DCIS), express the programmed cell death (PCD) inhibitors BCL2 and BCLXL. Therefore, molecular genetic manipulation to down regulate BCL2 and BCLXL expression may allow these premalignant lesions to reenter the PCD pathway prior to transformation to invasive breast cancer. In this study we proposed to determine if down-regulation of BCL2 and/or BCLXL expression would induce programmed cell death alone or in concert with tamoxifen to enhance its cytotoxic effect. We first determined the expression pattern of both BCL2 and BCLXL by Western blot in novel primary explant cell lines we established by

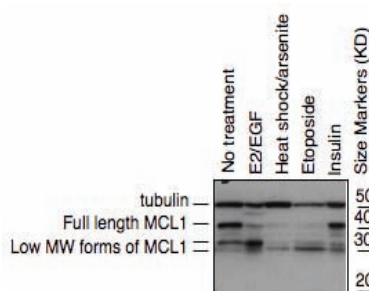


Figure 1. Expression of Low MW Forms of MCL1 in MCF-7 Cells Exposed to Various Agents

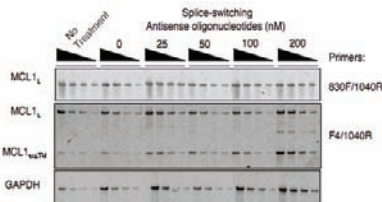


Figure 2. Induction of Low MW Forms of MCL1 in the Presence of Splice-switching Antisense Oligonucleotides

direct culture from human ductal carcinoma in situ tissue (JL-DCIS3B) and normal breast reduction tissue (JL-BRL23). We observed that the JL-DCIS3B primary explant cell line expressed both BCL2 and BCLXL while the normal breast tissue cell line JL-BRL23 did not express detectable levels of either protein. Next, we verified by Western blot analysis that 24 h liposomal transient transfection of JL-DCIS3B primary explant cells with the bi-specific antisense bcl-2/bcl-xl oligonucleotide down-regulated both BCL2 and BCLXL. Finally, we treated the JL-DCIS3B cells with the bi-specific antisense bcl-2/bcl-xl oligonucleotide alone, with physiological preventive doses of tamoxifen, or in combination to determine which had the highest induction of PCD. What we observed was that treatment with the bi-specific antisense bcl-2/bcl-xl oligonucleotide alone decreased BCL2 and BCLXL protein expression and induced programmed cell death, as measured by caspase activation and apoptotic body formation. Furthermore, treatment with tamoxifen alone did not induce programmed cell death and did not increase the amount of induction in combination with the antisense oligonucleotide. These data using primary explant cells suggest the potential of antisense bcl-2/bcl-xl oligonucleotide treatment alone, although more likely in combination with other conventional therapies, in preventing premalignant breast lesions from progressing to a malignancy by allowing these lesions to enter programmed cell death.

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P29-8: ANTI-TUMOR ACTION OF THE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 (IGFBP-3) AND IGFBP-3 RECEPTOR AXIS IN HUMAN BREAST CANCER

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The insulin-like growth factor (IGF) system is a critical regulator of the growth and differentiation of many tissues and organ systems. The IGF system consists of the IGF-I and IGF-II ligands, IGF-I and IGF-II receptors and IGF binding proteins (IGFBPs). Recently, the importance of the IGF system in a variety of human cancers has been addressed in large prospective studies by demonstrating a strong correlation between high IGF-I/low IGFBP-3 levels in the circulation and increased risk of cancer. Moreover, the involvement of IGFBP-3 in cell growth inhibition and induction of apoptosis in breast, prostate, colon and lung cancers has been demonstrated, although the specific mechanisms involved are unclear.

Recently we have demonstrated that IGFBP-3 induces apoptosis in an IGF-independent manner through the activation of caspases involved in a death receptor-mediated pathway in MCF-7 human breast cancer cells. Induction of IGFBP-3 inhibited DNA synthesis in an IGF-IGF receptor axis-independent fashion and resulted in the subsequent induction of apoptosis and an increase in caspase activity. Similar results were obtained when cells were transfected with GGG-IGFBP-3, an IGFBP-3 mutant unable to bind IGFs, corroborating the IGF-independent action of IGFBP-3. We have further identified a membrane protein that specifically binds to IGFBP-3. Subsequent functional studies indicate that this membrane protein may be a functional IGFBP-3 receptor (IGFBP-3R). The IGFBP-3R binds specifically to IGFBP-3, but not other IGFBP species. Over-expression of IGFBP-3R using an adenovirus gene transfer technique resulted in significant growth inhibition via induction of apoptosis in human cancer cells including breast, lung, colon and prostate cancer cells. Molecular and biochemical studies revealed that activation of the IGFBP-3/IGFBP-3R axis results in activation of caspases, in particular, caspase 8 and caspase 3, in these cancer cells, thereby suppressing cancer cell growth. Collectively, these observations suggest that the IGFBP-3/IGFBP-3R axis represents a new class of apoptosis signaling cascade that appears to be impaired in cancer cells. Thus, the IGFBP-3/IGFBP-3R axis may provide novel therapeutic targets for improved cancer treatment.

Together, these data demonstrate that the putative IGFBP-3R constitutes a new class of mammalian cell-death receptor, which mediates IGFBP-3-induced apoptosis by activating caspases as observed in human breast cancer cells. Thus, the IGFBP-3/IGFBP-3R axis may provide novel therapeutic targets for improved breast cancer treatment.

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P29-9: TARGETING RASMAPK HUMAN CANCER CELLS BY LEUCINE DEPRIVATION AS A NOVEL THERAPEUTIC STRATEGY

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The efficiency and fidelity of regulating cell functions in accordance to the nutrient availability is essential for cell survival. In vivo microenvironments of solid tumors are representative nutrient-depleted cell growth conditions and the tumor vasculature plays a critical role as a local nutrient supply, providing the rationale for the anti-

angiogenesis approach as a cancer therapy. However, the specificity of the nutrient molecule that is required to be deprived of to induce tumor regression remains largely unaddressed. We here report the AADIA (Amino Acid Deprivation Induced Apoptosis) phenotype of human cancer cells and the specific effect of the leucine deprivation on the induction of programmed cell death of human cancer cells. We initially surveyed various types of human cancer cells, including breast cancer cells, for their survival responses and identified a group of essential amino acids that activate the caspase-dependent programmed cell death when cells are deprived of them. While the deprivations of each essential amino acid arrest cell proliferation, accompanied with a dramatic downregulation of cyclin D1 protein, only the deprivations of a group of essential amino acid (Leucine, Methionine, Glutamine, Arginine, Threonine, Valine) induce apoptosis and the leucine deprivation has the strongest and broad effect over a majority of the responsive human cancer cell lines. To understand the underlying molecular mechanisms and cellular pathways responsible for this LAADIA (Leucine Amino Acid Deprivation Induced Apoptosis) phenotype, we perturbed signaling pathways by using the pharmacological inhibitors of known protein kinases and identified that specific MEK inhibitors strongly prevent the LAADIA. On the other hand, using the engineered human cancer lines, originally derived from immortalized human epithelial cells, that has either the activated RasG12V mutation or the constitutively active MEK1 mutation, we could demonstrate that the introduction of mutations activating the Ras-MEK axis of the Ras signaling pathway highly sensitizes cells to the LAADIA. Finally, we developed a novel real-time reporter for autophagy, a cellular pathway responding to the nutrient status. By using the autophagy reporter assay, we demonstrated that the leucine deprivation has significant effects on the regulation of autophagy that tightly correlates with the induction of the LAADIA. These findings demonstrate an unexplored interplay between the deprivation of a specific nutrient and the regulation of autophagy to determine cell fate. These results also suggest a novel strategy for the current nutrient-restriction based anti-tumor angiogenesis therapy.

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P29-10: MECHANISMS OF TMS (2,4,3',5'-TETRAMETHOXYSTILBENE) INDUCED APOPTOSIS IN BREAST CANCER CELLS

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A substantial fraction of postmenopausal women relapse and die of hormone-dependent breast cancer even though they initially responded objectively to Tamoxifen or Aromatase Inhibitors (AIs). Research over the past decade has focused on learning why patients develop resistance to hormonal therapies. These investigations demonstrated that breast cancer cells upregulate growth factor signaling pathways (EGF-R/HER 2/MAPK/PI-3K/mTOR) that render cells resistant to standard endocrine therapies. This knowledge has led to the use of several agents to block these signal transduction pathways such as MAPK, Her2 (Herceptin) and EGFR inhibitors (Iressa, Gleevec), but so far these therapies have not been sufficient to control metastatic breast cancer.

We hypothesize that killing breast cancer cells by apoptosis may be a more effective strategy. We are currently investigating alternative agents that can effectively kill hormone-resistant breast cancer cells. One promising agent is TMS (2,4,3',5'-tetramethoxystilbene), a derivative of the herbal compounds Rhapontigenin and Resveratrol. TMS is an inhibitor of cytochrome P450 1B1 and is capable of inhibiting tubulin polymerization and blocking the cell cycle at the G2/M phase. The goal of our study was to elucidate the molecular mechanisms contributing to TMS-invoked breast cancer cell death.

We found that the JNK/SAPK stress response pathway was activated. Bim, a microtubule associated protein bound to the dynein motor complex, was activated via JNK, released from microtubules and moved to the mitochondria. MCF-7 cells deficient for Bim exhibited a reduction in TMS induced apoptosis, providing evidence that Bim is causally involved. Mitochondrial outer membrane permeabilization (MOMP), induced by TMS in wild-type cells, was severely reduced in Bim-deficient cells. In addition, Bax was cleaved from the p21 form to the pro-apoptotic p18 form. Fractionation studies showed that TMS caused the release of cytochrome c, Smac/Diablo, apoptosis inducing factor (AIF) and endonuclease G from the mitochondria. Consistent with this, transmission electron microscopic (TEM) analysis demonstrated that mitochondrial cristae were damaged in TMS-treated cells. We found that the death was predominantly caspase independent and associated with the translocation of AIF and endonuclease G to the nucleus and changes in the chromatin structure, as observed by TEM in dying cells. Our findings demonstrate that TMS uses the JNK/stress pathway and mitochondrial death pathway to cause apoptosis of breast cancer cells. This knowledge will help us to develop better treatment strategies for hormone-resistant breast cancers.

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P29-11: FIBROBLAST GROWTH FACTOR BINDING PROTEIN EXPRESSION RESULTS IN INCREASED APOPTOSIS IN MOUSE MAMMARY GLANDS

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Fibroblast growth factors (FGFs) are vital modulators of mammary gland development as well as tumor angiogenesis. They play a large role in vascular formation, body axis patterning, cell migration, and organ branching. The complex FGF network involves over 22 distinct members that signal through 4 receptors to activate 3 major signaling pathways.

During embryonic development, a limited rudimentary mammary ductal tree is formed at the site of the nipple. The ovarian secretion of estrogen and progesterone occurs during puberty, resulting in ductal elongation throughout the mammary fat pad. This elongation is regulated by estradiol and the estrogen receptor α (ER α). Repeated cycles of ovarian stimulation result in further tertiary branching as the ductal tree is filled out. Steroid hormones have been shown to regulate multiple growth factors including multiple FGFs. FGF1, 2, 4, and 7 have been detected in various compartments of the mammary gland, and mutations in FGFR2 limited lobuloalveolar development during pregnancy indicating that FGF plays a role in normal mammary gland biology. More importantly, alterations in FGF signaling can lead to inappropriate cell behavior or pathology. In the murine mammary gland, FGF3, FGF4, and FGF8 have been identified as oncogenes after proviral insertion of mouse mammary tumor virus. Human breast cancer has also shown elevated levels of FGF8 as well as the amplification of FGFR1, FGFR2, and FGFR4. Progression of malignancy in mammary tumors shows a loss of FGF2, FGF7, and FGF10 expression while FGF1, FGF3, and FGF4 are upregulated.

A secreted protein, BP, acts as a chaperone molecule that binds and releases FGF from its customary site of storage in the extracellular matrix. BP has been shown to act as an angiogenic switch in tumorigenesis. In its role as a modulator of FGF, we hypothesized that BP would have an impact on normal mammary gland biology possibly leading to increased tumorigenesis. Using a tetracycline-inducible transgenic mouse model, we chronically overexpressed BP during various developmental stages of mouse mammary gland formation. We observed a significant decrease in tertiary branching in adult, fully formed mammary glands but not in pubertal mice where the mammary gland is still developing as shown by whole-mount analysis. Further experiments including immunohistochemistry showed this was due to increased apoptosis, which was evident after 1 week of induced expression but dropped to background levels after 30 days of BP expression. Decreased branching is not observed in mammary gland that has overexpressed hBP for 1 week, indicating that the decrease in branching occurs sometime between 1 week of induction and 1 month. FGF2 has been shown to induce apoptosis when over expressed in breast cancer cell lines, and low levels of FGF2 are associated with a more malignant phenotype in human breast cancer. Overexpressing BP may activate FGF2 to increase apoptosis in the mammary gland thereby inhibiting tumorigenesis rather than acting solely as an angiogenic switch to enhance tumor progression. Future directions will include BP transgenic mouse crosses with HER2/neu mice to evaluate the impact of BP on a breast cancer model.

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P29-12: APOPTOTIC MECHANISMS AND PATHWAYS ALTERED BY Myc IN MAMMARY EPITHELIAL MORPHOGENESIS

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Myc is a mammalian transcription factor that regulates transcription of a diverse network of target genes involved in cell growth, proliferation, differentiation, and apoptosis. In normal cells, Myc responds to both growth factor and cell death signaling to balance proliferation and apoptosis. In malignant cells, deregulation of Myc impairs apoptosis and leads to uncontrolled proliferation. Myc is frequently amplified and overexpressed in breast cancer, but its effects on proliferation, apoptosis, and therapeutic response are still poorly understood. The purpose of this research is to define the mechanisms and pathways relevant to Myc-induced apoptosis in the breast and determine how deregulation of Myc overcomes apoptosis as a barrier to breast cancer.

Our research utilizes a three-dimensional (3D) tissue culture model of breast ductal epithelia. In this system, non-transformed human mammary epithelial cells (MCF10A cells) are grown in Matrigel (a basement membrane culture matrix) to form 3D spheres (acini) that recapitulate the polarity of the human breast duct. As the cells establish the polarity of the acini, apoptosis selectively destroys cells in the interior of each sphere to create a hollow lumen. Apoptosis in the lumen requires Bim, a pro-apoptotic Bcl-2 family member that is regulated by Myc. Disruption of critical resi-

dues in the transcriptional activation domain of Myc prevent Myc from inducing apoptosis via Bim.

The objectives of this research are to (1) determine how Myc regulates Bim, a pro-apoptotic Bcl-2 family member that regulates mitochondrial death pathways, (2) probe which apoptotic effector pathways are required for Myc-induced apoptosis, (3) use mutational analysis of Myc to determine which structural elements of Myc are required for its ability to induce Bim-driven apoptosis, and (4) probe the connection between Myc and collaborating signaling pathways using shRNA-mediated knock-down of gene expression in the context of a 3D breast cancer model. In this system, Myc positively regulates Bim expression, proliferation, and apoptosis throughout the development of MCF10A cells grown in Matrigel to form 3D acini.

Breast cancers in which Myc are overexpressed must overcome the ability of Myc to promote apoptosis through Bim either by quantitatively or qualitatively regulating Myc function. Understanding the normal function of Myc in epithelial morphogenesis and how deregulation of Myc leads to pathogenic mechanisms of Myc-induced breast cancer will identify new targets for cancer therapy and potential therapeutic opportunities to restore Myc-induced apoptosis in malignant cells.

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P29-13: THE ROLE OF X-LINKED INHIBITOR OF APOPTOSIS IN BREAST CANCER DEVELOPMENT AND PROGRESSION

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Introduction: Apoptosis is a process of cell death that is regulated by both pro- and anti-apoptotic proteins. A hallmark of cancerous cells is the acquired ability to block this process of programmed cell death, facilitating tumor growth and resistance to chemotherapy. Potential cancer therapeutics are in development to specifically target the apoptotic pathway and promote programmed cell death. One promising molecular candidate is x-linked inhibitor of apoptosis (XIAP), an anti-apoptotic protein that is abundant in breast carcinoma specimens. XIAP directly inhibits caspases-3, -7, and -9, proteases that are responsible cleaving specific cellular contents during apoptosis. We hypothesized that overexpression of XIAP might contribute to both tumorigenesis and progression. Our studies investigate the effect of loss of XIAP in breast carcinoma cell lines and mouse models of breast cancer to understand how XIAP contributes to breast cancer.

Methods: Plasmids: Oligonucleotides encoding short hairpin RNAs targeting XIAP or control sequences were annealed and ligated into pSuper. The H1 promoter and shRNA fragment were cloned into pFG12. XIAP cDNA was altered by site-directed mutagenesis to contain silent mutations in the RNA interference target sequence for expression in the presence of shRNA. Similarly, site-directed mutagenesis was also used to alter critical residues for caspase inhibition (D148A and W310A) and RING activity (H467A). Cell culture: MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% FBS and 2 mM glutamax, and 293T cells were grown in DMEM, 10% FBS and 2 mM glutamax. To package self-inactivating lentiviruses, 293T cells were transfected with 5 μ g each of pFG12, pRSV-REV, pRRE, and pVSV-G with calcium phosphate for 7 hours. Thirty-six hours post-transfection, viral supernatant was filtered onto target cells. Xenograft tumor growth: MDA-MB-231 cells were resuspended in PBS to 3×10^7 cells/mL and 100 μ L was subcutaneously injected into 5-week old athymic nude mice (Charles River Laboratories, Cambridge, Massachusetts) bilaterally in the dorsal posterior quadrants. Tumor growth was monitored by caliper measurement of two perpendicular dimensions, estimating tumor mass as $a^2 \cdot b/2$, where a is the shorter measurement.

Results: XIAP protein levels were suppressed in human breast adenocarcinoma MDA-MB-231 by RNA interference. In vitro, loss of XIAP did not affect population doubling time in 10% serum or anchorage-independent growth in soft agar. However, loss of XIAP does sensitize MDA-MB-231 cells to pro-apoptotic stimuli. In vivo, MDA-MB-231 cells form tumors in immunodeficient mice, and experiments to determine the tumorigenicity of XIAP-deficient MDA-MB-231 cells are ongoing.

Conclusions: Blockade of programmed cell death is believed to be a critical event during both initial tumor development and later progression. Confirming previous observations, XIAP protects MDA-MB-231 cells against apoptosis induced by extracellular stimuli and certain chemotherapeutics. We are currently investigating the role of XIAP in mouse models of breast cancer. Small molecules and antisense oligonucleotides that antagonize XIAP are being pursued in preclinical and clinical trials. Examining the effect of loss of XIAP in breast cancer models in vivo will provide insight into the molecular functions of XIAP that are relevant to its role in breast cancer development and progression.

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P29-14: REGULATION OF PHOSPHATIDYL SERINE ASYMMETRY BY CA²⁺-DEPENDENT MEMBRANE FUSION DURING APOPTOSIS OF BREAST CANCER CELLS

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One of the hallmarks of apoptotic cells is the appearance of phosphatidylserine (PS) at the cell surface that occurs as a result of its redistribution from the cells inner-to-outer membrane leaflet. Our studies address the apoptosis-dependent mechanisms that regulate PS translocation across the plasma membrane bilayer of breast carcinoma cells. In contrast to red blood cells that do not contain intracellular organelles, treatment of breast cancer cells with sulfhydryl-reactive reagents results in the expression of PS that is accompanied by release of Ca²⁺ from intracellular stores to the cytosol. Attempts to empty endoplasmic reticulum (ER) and mitochondrial Ca²⁺ stores failed to result in PS exposure suggesting that the Ca²⁺ required for this process comes from other intracellular organelles or from extracellular sources. We therefore initiated a series of experiments to determine the source of Ca²⁺ required for PS exposure. Fluorescence microscopy of Rhod-2 AM-labeled cells revealed large intracellular vesicles that were distinct from ER, mitochondria and Golgi. A series of experiments employing rhodamine-phosphatidylethanolamine-labeled plasma membranes, fluorescein-labeled high molecular weight dextrans and calcium-green, unequivocally proved these structures were endocytotic vesicles that released their Ca²⁺-containing contents into the cytosol upon apoptosis. Importantly, this process was accompanied by fusion of the labeled vesicles with the plasma membrane. The requirement for apoptosis could be circumvented by treating the cells with Ca²⁺ ionophore/Ca²⁺ that resulted in fusion of endocytotic vesicles, ER, golgi and mitochondria membranes with the plasma membrane. This suggests that the redistribution of PS during apoptosis occurs by a Ca²⁺-dependent mechanism that is independent of DNA fragmentation and caspase activation.

In summary our data indicate that extensive membrane rearrangements and inter-organelle fusion occur through a mechanism that involves specific elevations in cytosolic Ca²⁺ levels during apoptosis that results in the reorientation of PS from the cells inner-to-outer membrane leaflet. This work provides the basis toward understanding the molecular events that regulate the elimination of errant cells. The identification of candidate molecules that regulate PS externalization will facilitate the development of specific targeted therapeutic strategies.

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P29-15: BCL2 FAMILY FUNCTION IN ANTIESTROGEN-RESISTANT BREAST CANCER CELLS

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Tamoxifen (TAM), one of the most widely used antiestrogens in breast cancer therapy, has demonstrated the ability to improve disease-free as well as overall survival in most estrogen receptor-positive (ER+) breast cancer patients. The steroidal antiestrogen ICI 182,780 (ICI; Fulvestrant) is a pure antagonist of the ER and is often effective as a second-line treatment for women who do not respond to TAM. Unfortunately, the development of resistance to both of these antiestrogen therapies is an overwhelming concern in the clinic. It is widely known that defects in the apoptotic pathway can lead to drug resistance, and both TAM and ICI can induce apoptosis in breast cancer cells. However, the precise mechanism by which impaired apoptosis contributes to antiestrogen resistance remains unknown. We and others have demonstrated that antiestrogens can regulate apoptosis by altering the expression of BCL2 family members, but the effects of TAM and ICI are not always consistent. Therefore, we hypothesize that BCL2 family members play distinct roles in antiestrogen-resistant breast cancer that are determined by the type of antiestrogen to which the cells display resistance.

To test this hypothesis, we used two antiestrogen-sensitive and -resistant breast cancer cell line pairs: ICI-resistant MCF7/LCC9 cells and their sensitive controls MCF7/LCC1 and the TAM-resistant MCF7/RR cells and their sensitive controls MCF7. Baseline expression of the antiapoptotic proteins Bcl-2 and Bcl-w is increased in the ICI-resistant LCC9 cells as compared to LCC1. However, there is no change in these antiapoptotic BCL2 family genes in the TAM-resistant MCF7/RR cell line as compared to MCF7 cells. In contrast, TAM-resistant MCF7/RR cells express significantly lower levels of the pro-apoptotic proteins Bax and Bad relative to MCF7 cells while there is no difference in the expression of these molecules between ICI-resistant LCC9 cells and -sensitive LCC1 cells. We have also shown that TAM and ICI no longer affect expression of these BCL2 family members in resistant breast cancer cells. Bcl-2 expression is not reduced by ICI in the resistant LCC9 cells as compared to LCC1, while Bax induction by TAM is delayed in MCF7/RR cells relative to MCF7. Finally we have determined, with the use of transiently transfected Bcl-2 siRNA, that knock-down of Bcl-2 in the LCC9 cells increases apoptotic cell death when treated with antiestrogens.

In conclusion, our data suggest that antiapoptotic BCL2 family members play a role in ICI resistance and proapoptotic family members play a role in TAM resistance. Our

data also suggest that altering Bcl-2 expression may restore antiestrogen sensitivity in resistant cell lines. In future studies, we will further identify specific changes in Bcl-2 family member transcription and regulation by identifying transcription factors that may play a role in antiestrogen resistance.

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P29-16: THE UNFOLDED PROTEIN RESPONSE PATHWAY: A POTENTIAL TARGET FOR DRUG DEVELOPMENT IN BREAST CANCER

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The unfolded protein response (UPR) activates transcriptional programs that enable cells to survive stress. Key players of the human UPR are IRE1 and XBP-1. IRE1 senses accumulation of unfolded proteins in endoplasmic reticulum (ER) and transduces this signal by removal of an intron from the XBP-1 mRNA. The resulting frameshift yields an active transcription factor. Loss of XBP1 inhibits tumor growth implicating it as important for survival. We have data that the UPR is activated in a large fraction of breast cancer cell lines and tumors. Expression profiling of XBP-1 in 50 human breast cancer cell lines representing all molecular subtypes revealed that it was expressed at higher levels than the control breast epithelial cell line in all luminal cell lines. XBP-1 expression was relatively decreased in all of the ERBB2 and basal B cell lines, and decreased in 11/13 of basal A cell lines. We also analyzed expression data from specimens of each major subclass of breast cancer. XBP-1 was over-expressed in 26/42 of the luminal, 27/39 of the basal A, but only in 2/42 basal B type molecular subtypes. Given the likely protective role of the UPR in breast cancer, we will screen small molecule libraries for UPR inhibitors and test whether UPR inhibition reduces cell viability. We will develop a new class of anticancer drugs for breast cancer.

Aim 1. To determine the effect of UPR inhibition on survival of breast cancer cells, we will test siRNA against Ire-1 and XBP-1, to establish causality between UPR activation and cancer cell survival. We will test the siRNA also on breast cancer cell survival with known therapeutic drugs against breast cancer.

Aim 2. We will use high throughput screening (HTPS) with selective chemical libraries to identify chemical inhibitors of IRE1.

Aim 3. We will determine if UPR inhibitors impact on survival of breast cancer cells, and if our inhibitors enhance the cytotoxicity of anthracycline and taxane chemotherapeutic drugs.

We have developed assays that allow screening for chemical compounds. In one, an ATP analog, 1NM-PP1 (designed/synthesized at UCSF), that binds to the ATP binding pocket of a variety of proteins, including IRE1, inhibited splicing in cells in which the UPR was activated. We have >40 other 1NM-PP1-like molecules and will determine if each inhibits IRE1. We also developed a HTPS assay using a fusion gene linking the XBP-1 and green fluorescent protein (GFP) mRNAs. Activation of IRE1 cleaves the XBPu-GFP mRNA resulting in cessation of GFP synthesis. Also, we have developed a yeast-based assay for HTPS of UPR inhibitors. We induce a UPR in yeast bearing human IRE1 and use a colorimetric assay for UPR activation or inhibition. The UCSF Small Molecule Discovery Center has screening libraries containing >150,000 small molecules with 70,000 kinase-targeted compounds comprised of substituted heterocyclic compounds expected to compete for the kinase ATP binding site. Besides HTPS, compounds predicted to bind to functional sites on IRE1 based on atomic structure are being identified in silico and tested. The crystal structures of the yeast and human Ire-1 luminal domain are used to dock in silico chemical libraries such as the ZINC database. UPR inhibitors are further derivatized in iterative rounds of in silico docking and in vivo assays. We will solve the crystal structures of the Ire-1 luminal domain complexed with the strongest chemical inhibitors to refine docking parameters.

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P29-17: TOLL-LIKE RECEPTOR 9 AGONISTS STIMULATE BREAST CANCER INVASION

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Background: Toll-like receptor 9 (TLR9) belongs to the innate immune system and it recognizes microbial and vertebrate DNA. TLR9 ligands evoke an immune reaction including the production of pro-inflammatory cytokines. Intracellular signaling mediators of TLR9 include MyD88 and TRAF6. In addition to mediating immunity, Toll, the Drosophila analog of mammalian TLRs, also mediates dorso-ventral patterning

during embryogenesis. We showed previously that treatment with TLR9 agonistic, CpG-sequence containing oligonucleotides (CpG-ODN) induces MMP-13-mediated invasion in TLR9-expressing human breast cancer cell lines. The aim of this study was to further characterize the role of the TLR9 pathway in this process.

Methods: Human MDA-MB-231 breast cancer cells were stably transfected with a dominant negative (DN) TRAF6 or a control cDNA. Peritoneal macrophages were isolated from wild type, TLR9 $-/-$, or MyD88 $-/-$ mice. CpG-ODN sequence modifications were designed with the molecular modeling (MFOLD) software. Cellular invasion was studied with Matrigel assays in vitro. TLR9 expression in clinical specimens was studied with immunohistochemistry.

Results: CpG-ODN induce invasion in wild type and MyD88 $-/-$ macrophages. This effect is significantly inhibited in TLR9 $-/-$ macrophages and in MDA-MB-231 cells stably expressing DN TRAF6. CpG-ODN sequence modifications affecting the stem-loop secondary structure, influenced the invasion-inducing effect in MDA-MB-231 cells, while methylation of the cytosine residues in the parent CpG-ODN did not. In clinical breast cancer samples and normal breast tissue, TLR9 staining localized in epithelial cells. The mean epithelial TLR9 staining intensity was significantly increased in the breast cancer specimens, as compared with normal breast tissue. In another breast cancer cohort (n=119), TLR9 staining intensity in the primary tumors was compared to other biological parameters of the tumor. TLR9 staining was detected in ~97% of the specimens. TLR9 staining intensity was significantly higher (p<0.01) in the estrogen receptor-negative (ER-) tumors, as compared with highly ER positive (ER++) tumors. TLR9 staining intensity was independent of progesterone receptor, Her2/neu expression, and histological subtype of the tumor.

Conclusions and Potential Impact: TLR9 is frequently expressed in breast cancer. TLR9-agonist induced cellular invasion is mediated via TLR9 and TRAF6, independent of MyD88. The structure and/or stability of DNA may influence the induction of TLR9-mediated invasion in breast cancer. TLR9-mediated invasion may represent a novel pathway of breast cancer invasion, which may be significant especially in ER-breast tumors. Further studies are needed to identify physiological TLR9 agonists that activate this invasive pathway in breast cancer. If TLR9 proves significant in the pathogenesis of breast cancer then the efficacy of TLR9 inhibitors, such as chloroquine, should be tested in breast cancer patients.

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P29-18: NQO1-DEPENDENT REACTIVE OXYGEN SPECIES ARE NECESSARY, NOT SUFFICIENT, FOR β -LAPACHONE-MEDIATED CELL DEATH

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β -lapachone is a novel chemotherapeutic and radiosensitizing agent that targets cancer versus normal cells through endogenous overexpression (5- to 20-fold) of NAD(P)H: quinone oxidoreductase 1 (NQO1). A deficiency, or inhibition, of NQO1 renders cells resistant to β -lapachone. Since NQO1 is induced in cancer cells after ionizing or UV radiation or after tamoxifen treatments, β -lapachone may be used to treat specific cancers with elevated/inducible NQO1 levels (e.g., breast, non-small cell lung, pancreas, colon, and prostate cancers). Previously, we showed that β -lapachone-induced caspase- and p53-independent apoptosis was dependent on endoplasmic reticulum calcium release and BAPTA-sensitive poly(ADP-ribose) polymerase-1 (PARP-1) hyperactivation. Initiating events to induce this novel cell death pathway were not elucidated.

NQO1 reduced β -lapachone to an unstable hydroquinone that rapidly underwent a two-step oxidation back to β -lapachone. Futile cycling of β -lapachone by NQO1 consumed oxygen and generated reactive oxygen species (ROS). Each mole of β -lapachone cycled by NQO1 consumed ~445 moles of oxygen within 30 minutes. Inhibition of this robust redox cycling required high N-acetyl-L-cysteine concentrations and/or overexpressed manganese superoxide dismutase coupled with NQO1 modulation by dicoumarol. ROS formed in β -lapachone-treated NQO1+ cells led to DNA damage, PARP-1 hyperactivation, NAD⁺/ATP depletion, and cell death. ROS generation occurred before calcium increases. Since sublethal doses of β -lapachone caused ROS formation and DNA damage, but not PARP-1 hyperactivation, ROS appear necessary, but not sufficient, to induce cell death. Instead, a threshold level of DNA damage caused by β -lapachone-induced ROS was necessary for PARP-1 hyperactivation. PARP-1 hyperactivation was therefore sufficient for β -lap-induced cell death in NQO1+ cancer cells. This mechanism can be exploited by combined cancer therapy with DNA damaging agents. We are currently exploring the roles of b5R and p450R in β -lapachone cytotoxicity, as well as using free radical scavengers and/or NQO1 inhibitors as antidotes to β -lapachone to ameliorate unwanted normal tissue toxicities. Finally, we have developed nanoparticles to deliver β -lapachone specifically to tumor tissue and are examining the efficacy of these devices at the present time.

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P29-19: MODULATING COMPLEMENT TO ENHANCE APOPTOSIS-BASED THERAPY OF BREAST CANCER

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Many conventional therapies of cancer (chemotherapy, radiation, and immunotherapy) involve the induction of tumor cell apoptosis, and gene therapy approaches for the delivery of apoptosis-based therapeutics currently under investigation also offer promise for the treatment of cancer. The effectiveness of apoptosis-inducing therapy of cancer can be significantly enhanced by an initial local inflammatory reaction at the tumor site and a subsequent specific immune response. We are investigating whether modulating complement activation during apoptosis-based therapy, which is predicted to inhibit phagocytic uptake of apoptotic cells/bodies, enhances an inflammatory/innate immune response within the tumor environment. We are also investigating whether modulating complement in combination with apoptosis-based therapy will augment or even trigger specific immunity against a tumor to which the host is normally tolerant. In initial studies, we introduced the pro-apoptotic gene, Fas ligand (FasL), directly into established subcutaneous EO771 (murine breast cancer cell line) tumors growing on the flank of female C57BL/6 mice using a recombinant adenovirus (rAd) gene therapy vector. Mice were separated into two groups, and one group received iv injections of CR2-Crry, a mouse complement inhibitor, every 2 days until sacrifice at day 14. There was a significant decrease in tumor volume in both groups of mice (i.e., mice treated with rAdFasL only and mice treated with rAdFasL+CR2-Crry). However, there was a significantly higher number of apoptotic cells in the remaining tumor in mice that had received CR2-Crry. Based on the known important role of complement in phagocytic uptake of apoptotic cells, this result indicates complement inhibition interferes with the clearance of apoptotic cells induced by the AdFasL therapy. This project was only recently initiated, and we will report further on the role of complement in modulating innate and adaptive responses subsequent to the rAdFasL-based therapy at the meeting. Using the EO771 mouse model with rAdFasL and CR2-Crry treatment, we are currently investigating the effect of complement inhibition on systemic and tumor cytokine levels, tumor infiltration of immune cells, and antibody and T cell response to EO771 cells.

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P29-20: THE ROLE OF THE Rad9-Rad1-Hus1 COMPLEX IN DNA DAMAGE-INDUCED APOPTOSIS

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The Rad9-Rad1-Hus1 (9-1-1) cell cycle checkpoint complex plays a key role in the response to DNA damage. Cells that have a defective 9-1-1 complex, as a result of loss of one of the members, have been shown to be extremely sensitive to genotoxic stresses and thus are hypersensitive to apoptosis. This suggests that the 9-1-1 complex may play a role in conferring resistance to apoptosis in response to certain types of DNA damage. The acquired resistance of breast cancer cells to traditional chemotherapeutic agents is one of the most difficult challenges in the treatment of breast cancer. Therefore, determining the mechanism linking the loss of a functional 9-1-1 complex to the cell death machinery would give insight for the development of strategies that would sensitize otherwise resistant breast cancer cells to DNA damaging agents. In this study, we report that disruption of the 9-1-1 complex sensitizes cells to the topoisomerase II poison, etoposide, through upregulation of the pro-apoptotic, BH3-only, proteins Bim and Puma. Etoposide treatment significantly induced the expression of these BH3-only proteins in Hus1-null mouse embryonic fibroblast (MEF) cells, as compared to Hus1-positive cells. Restoration of Hus1 expression in Hus1-null cells suppressed both Bim and Puma induction in response to etoposide treatment. Similarly, treatment with etoposide resulted in a greater induction of these BH3-only proteins in human breast cancer cells in which the 9-1-1 complex was disrupted. Furthermore, inhibition of either Bim or Puma expression in Hus1-null cells conferred significant resistance to DNA damage-induced apoptosis. Knockdown of both Bim and Puma resulted in a greater resistance, suggesting that Bim and Puma collaborate in sensitizing Hus1-null cells to etoposide treatment. We also found that, in Hus1-positive MEFs, Rad9 was mostly localized in the nucleus where it bound to chromatin in response to etoposide-induced DNA damage. In contrast, Rad9 was predominantly located in the cytoplasm of Hus1-null cells where it was found to interact with Bcl-2. Taken together, these data suggest that the 9-1-1 complex confers resistance to DNA damage not only by suppressing the induction of the BH3-only proteins, Bim and Puma, but also by keeping Rad9 in the nucleus and preventing its release into the cytosol where it could interact with and neutralize anti-apoptotic members of the Bcl-2 family.

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P29-21: ANALYZING GENE EXPRESSION DATA FOR p53 PATHWAY FUNCTION IDENTIFIES Bik AS AN INDICATOR OF POOR PROGNOSIS IN BREAST CANCER

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Loss of p53 tumor suppressor function occurs with such high frequency in human cancer that it clearly provides a strong selective advantage for tumors. However, this high frequency of p53 loss-of-function also poses a significant confounding factor for gene expression analysis from patient tumor samples. Since loss of p53 function compromises apoptotic signaling, such a tumor is relieved of any selective pressure to alter additional components of the apoptotic pathway. In this report, we determined whether novel apoptotic regulators of patient prognosis can be identified by filtering gene expression data for intact p53 function. Using expression of Reprimo and

GADD45 as indicators of p53 function, we identify Bik as a gene that is significantly downregulated in breast tumors from patients with poor prognosis. Bik mRNA and protein are strongly reduced in a panel of 11 human breast tumor cell lines, compared to nontumorigenic MCF10A cells. Bik expression is selectively induced by cell rounding and loss of Bik in tumor cell lines correlates with a resistance to apoptosis caused by cell rounding, or amorphosis. This significant downregulation of Bik would not have been apparent from gene expression data alone without focusing on patients whose tumors display indicators of a functional p53 pathway. Given the prevalence of p53 loss-of-function in human cancers and its powerful selective advantage, it will be important for future gene expression studies to account for p53 pathway function when assessing the prognostic significance of apoptosis genes.

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SIGNAL TRANSDUCTION I

Poster Session P30

P30-1: ERYTHROPOIETIN RECEPTOR EXPRESSION IN BREAST CANCER CELLS: ROLE IN EPO-MEDIATED ACTIVATION OF SIGNAL TRANSDUCTION AND CELLULAR PROLIFERATION AND MIGRATION

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The EPO receptor (EpoR) is expressed in breast cancer cells but its contribution to tumor progression and metastasis requires further characterization. We hypothesized that EpoR expression levels and activation in breast cancer cells may modulate cellular proliferation and migration. As a model, MCF-7 cells were engineered to stably express a constitutively active EpoR mutant R129C-EpoR. EPO treatment of untransfected MCF-7 cells—which express endogenous EpoR protein—induces increased tyrosine phosphorylation of multiple cellular proteins. EPO treatment leads to increased phosphorylation of JNK and ERK kinases but not JAK2, AKT or STAT5. Increased EPO-induced ERK phosphorylation is associated with 2.3 ± 0.3 -fold increase in ERK kinase enzymatic activity ($p < 0.05$). EPO had a minor effect on the proliferation of untransfected MCF-7 cells and significantly increased cellular migration in an in vitro assay compared to buffer-treated controls ($p < 0.05$). In the absence of EPO, R129C-EpoR expression resulted in 2-fold increased proliferation ($p < 0.01$, 3 single cell clones) and 1.5 ± 0.2 -fold increase in migration compared to vector controls ($p < 0.05$). EPO treatment significantly stimulated the migration of vector-transfected MCF-7 cells by 1.9 ± 0.2 -fold ($p < 0.001$). EPO treatment did not further enhance the increased basal proliferation and migration of R129C-EpoR expressing cells. In R129C-EpoR expressing MCF-7 clones, there was a significant increase in the basal phosphorylation of ERK (2.6 ± 0.4 -fold, average of 3 clones, $p < 0.05$) and AKT (1.6 ± 0.1 -fold, $p < 0.05$) compared to vector-transfected negative controls in the absence of EPO. No changes in the phosphorylation status of STAT5, JAK2 or p38 MAP kinases were observed in response to EPO treatment or under basal conditions. Whereas EPO did not induce the increased phosphorylation of AKT in untransfected or vector-transfected MCF-7 cells, in R129C-EpoR expressing cells, there was a significant, EPO dose-dependent increase in AKT phosphorylation as a result of EPO (3.29 ± 0.5 -fold, 3 clones, $p < 0.05$). In R129C-EpoR expressing cells treated with EPO, ERK phosphorylation increased significantly 2.7 ± 0.3 fold ($p < 0.001$) and JNK phosphorylation increased by 5.38 ± 1.6 fold ($p < 0.001$) compared to vector-transfected controls in a dose-dependent manner. Increased EPO-induced migration of vector-transfected cells was blocked by treatment with JNK kinase inhibitor SP600125. The increased migration of R129C-EpoR expressing cells in the absence of exogenous EPO was also blocked by JNK kinase inhibitor SP600125. These data indicate that (1) EPO activates the ERK kinases in MCF-7 breast cancer cells, (2) EPO promotes the migration of breast cancer cells through a JNK-kinase dependent signaling pathway, (3) expression of constitutively active R129C-EpoR promotes the proliferation and migration of breast cancer cells, (4) sensitivity to exogenous EPO is enhanced by R129C-EpoR expression leading to restoration of EPO-dependent AKT phosphorylation and increased phosphorylation of ERK and JNK kinases, (5) the biologic effects of EPO in breast cancer cells are mediated through non-JAK2-STAT5 axis-dependent signaling pathways, and (6) EpoR expression levels in breast cancer cells may modulate EPO-mediated intracellular signaling, proliferation, and migration responses.

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P30-2: PHOSPHORYLATION SITES THAT REGULATE c-Myc PROTEIN STABILITY AND ONCOGENIC POTENTIAL

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The c-Myc oncoprotein is overexpressed in around 70% of human breast tumors and animal models have demonstrated the potent nature of c-Myc in mammary tumorigenesis. Our research has helped to elucidate a complex signaling pathway that regulates c-Myc protein stability via two conserved phosphorylation sites, Threonine 58 and Serine 62, which could account for the high incidence of its overexpression, since c-Myc gene amplification is only reported in 15% of breast cancer. Under normal conditions, c-Myc protein levels are kept very low through its rapid ubiquitin-mediated degradation. Stimulatory signals transiently stabilize c-Myc through Ras-dependent phosphorylation of Serine 62 (S62). Subsequent phosphorylation of Threonine 58 (T58) by GSK-3 β destabilizes c-Myc through recruitment of the Pin1 prolyl-isomerase, which facilitates protein phosphatase 2A (PP2A)-mediated removal of the stabilizing phosphate at S62. PP2A is targeted to c-Myc by the B56 α regulatory subunit. T58 phosphorylated c-Myc is then recognized by the SCF^{Fbw7} E3 ubiquitin ligase, which polyubiquitinates c-Myc targeting it for degradation. Our recent research has revealed a role for the Axin scaffold protein in coordinating these enzymatic steps through the formation of a degradation complex for c-Myc containing GSK3 β , PP2A-B56 α and Pin1. We have found that deregulation of this signaling pathway occurs in breast cancer leading to aberrant stabilization and accumulation of c-Myc. Our analysis demonstrates that c-Myc is stabilized in multiple human breast cancer cell lines. Importantly, stabilized c-Myc has high S62 phosphorylation with low T58 phosphorylation. This phosphorylation pattern also occurs in some breast cancer samples compared to normal breast tissue. To study the effects of altering

c-Myc phosphorylation at T58 and S62 in vivo, we have engineered three c-myc knock-in strains of mice in which we can conditionally express either wild-type c-MycWT, the c-MycT58A phosphorylation mutant or the c-MycS62A phosphorylation mutant from the weak, but constitutively active ROSA promoter in response to Cre recombinase. We have crossed these three strains of mice with WAP-Cre transgenic mice for mammary specific expression. c-MycWT expression in this model is insufficient for mammary tumorigenesis due to the low level of expression so that c-Myc's proliferative potential is balanced by its apoptotic activity. In contrast, c-MycT58A shows increased oncogenic potential with decreased apoptotic activity compared to c-MycWT in this model. Specifically, c-MycT58A mice show substantially delayed involution due to inhibition of apoptosis. This is associated with decreased expression of the pro-apoptotic Bim protein and increased expression and phosphorylation of the anti-apoptotic Bcl2. c-MycT58A, but not c-MycWT mice develop mammary adenocarcinoma after 8-12 months of expression. In contrast, c-MycS62A appears to have reduced activity compared to c-MycWT in this model and mammary glands from c-MycS62A mice appear similar to control mice. These results have important implications regarding a new mechanism for regulating the oncogenic potential of c-Myc, where post-translational regulation of its stability is coupled to its tumorigenic activity. Moreover, this mechanism appears to be relevant to human breast cancer.

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P30-3: INTER-CELLULAR COMMUNICATION MODELS FOR EFFECTIVE AND REALISTIC BIOLOGICAL SIMULATIONS

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Current computational modeling has provided significant in-roads on describing biological systems. However, there is still a huge gap to bridge in simulation work to realistically model the development of the mammary gland and the origin of breast tumors. There has yet to be a simulation framework proposed that emphasizes signaling interaction between mammary epithelial cells and ECM, and the related modulating of hormonal signals. This is due to difficulties within existing simulation systems to handle the complex heterogeneous signaling interactions at both cellular and tissue levels.

Our goal is to research and develop an asynchronous simulation framework that models the dynamic behaviors of mammary components to understand how the interactions among them decide the development fate using our established techniques/platform of mobile multi-agent systems. Such work is particularly important in breast cancer research. The objectives for this project are to: develop a multi-agent system modeling the mammary gland; formulate the behavior of epithelia and myoepithelia cells as probabilistic decision makers that interact with ECM; develop a model for signaling within the tissue; and implement, evaluate, and validate the data reported.

We have researched and developed key components including the Distance Based Broadcast model and the asynchronous message passing paradigm. By doing so, we can effectively simulate paracrine signaling and various propagations for the long range. Current multi-agent systems communication models are quite limited in scope.

To provide initial validation, we considered *Dictyostelium discoideum*, a fundamental and well-studied cell system that is a slime mold that begins as a unicellular organism, but when starved, will become a multicellular organism or mound. Focusing on the starvation period, it undergoes cell differentiation and morphogenesis. The life cycle consists of aggregation, migration, and culmination phases.

In our validation, we focused on the aggregate pattern formation in early life cycle during starvation, which is circular with an observed average diameter of 0.0002 meters. One set of experiments varied cell density and showed that the circular pattern is more prevalent with large density and less apparent with smaller densities. We obtained further results comparing our communication models with a baseline model where all agents receive a signal with no regard to the distance between them and the sender (a common design in multi-agent systems). In this set of experiments, we held the density stable and considered the average radius of the circular patterns of aggregation versus the signal propagation distance. The baseline model produces the worst diameter results. Our approach produced average diameters of 0.0002 (the same value observed by experimental data) when the signal propagation distance is between 0.00025–0.00035. These tests show that even in fairly simple organisms, the need to represent inter-cellular signaling is critical toward effective simulation. We see this as a vital first step.

Without a doubt, simulation is a critical component toward understanding the progression of breast cancer. Our ultimate goal is to design a simulation system with sufficient details that it can be used to analyze the behavior of the mammary gland, evaluate different theories and models, and provide insights into the origin and progress of breast tumors.

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P30-4: BREAST CANCER CELLS RESPOND TO SEROTONIN SIGNALS

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Serotonin is primarily thought of as a neurotransmitter. Only 5% of the body's serotonin is in the central nervous system (where it is trapped by the blood-brain barrier); the vast majority is in the periphery. Throughout evolution, the dominant role of serotonin has been as a cell activator and as a factor for cellular differentiation. Our data demonstrate that the serotonergic pathways are functionally present in breast cancer cells. These data also suggest that serotonin may be acting as a primordial autocrine growth factor for maintaining the activation and proliferation of breast cancer cells.

For comparative studies, we have characterized the mRNA encoding the various serotonin receptors in MCF-10A cells (a line derived from healthy tissue), MCF-7 cells (an estrogen-dependent cell line derived from an adenocarcinoma), and BT483 cells (an estrogen-independent line derived from an invasive ductal tumor). The data were compared to normal, primary breast epithelial cells. All of the cells expressed mRNA encoding 5-HT receptors; however, the profile and relative mRNA levels were different for each of the cells. Thus, the "machinery" for responding to serotonergic signals was present in all of the cell types. The serum used to supplement tissue culture growth contains enough serotonin to saturate most of the 5-HT receptors. We charcoal-filtered the sera to grow the cells in the absence of exogenously added serotonin. All of the breast-derived cell lines changed their transcription profiles in response to the charcoal-filtered media. Because charcoal-filtering removes more than just serotonin, we added back serotonin to the media to find out if any of the transcriptional changes were related to the serotonin pathway. Each different cell line had a unique response to the added serotonin. There is clearly a feedback pathway in all of these cells that transcriptionally responds to the presence or absence of serotonin.

Highly selective inhibitors are available for most of the 5-HT receptors. In the BT483 cells, for example, inhibition of the 5-HT_{1B} receptor resulted in a loss of cell viability and the induction of programmed cell death. In all of the breast cancer cell lines tested, withdrawal of the serotonergic signaling results in cell cycle arrest and in the induction of apoptosis. Collectively, these data suggest that serotonergic pathways may offer new therapeutic targets in the design of treatment strategies for combating breast cancer.

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P30-5: ROLE OF THE TYROSINE PHOSPHATASE SHP-1 AND REGULATORY T CELLS IN BREAST CANCER

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Background and Objectives: The failure of immune surveillance by the body allowing uncontrolled proliferation of tumor cells has been linked to cancer development. Regulatory T cells (Treg) play a critical role in immune tolerance to self by suppressing immune responses to autoantigens. For a variety of cancers, including breast cancer increased Treg cell numbers have been reported. However despite the increasing appreciation of Treg cells, our mechanistic and molecular understanding of their development and function is still very limited.

The tyrosine phosphatase SHP-1 is a well-recognized negative regulator of T cell signaling that also affects the generation of Treg cells. Mice lacking SHP-1, so-called motheaten (me/me) mice, have increased numbers of CD4+CD25+Foxp3+ Treg cells. Interestingly, mice heterozygous for the me allele show a high occurrence of breast cancer. The overall goal is therefore to test the existence of a link between Treg cells, SHP-1 function, and tumor incidence.

Methodology and Results: To interfere with SHP-1 function in a cell lineage specific manner, we generated mice that carry dominant negative mutants of SHP-1 using the Cre/loxP system sites that allows tissue-specific expression upon crossing into Cre-expressing mouse strains. Analyses of mice with decreased SHP-1 activity in the T cell lineage showed an increase in the number of CD4+CD25+ Treg cells in lymph nodes and spleen compared to mice with wild-type SHP-1 activity indicating a T cell intrinsic effect of SHP-1 on Treg cell development. We are currently crossing these mice in different genetic backgrounds that are susceptible to mammary tumor development to assess whether increased Treg numbers contribute to the onset and development of breast cancer.

Since SHP-1 is a negative regulator of TCR-mediated signaling, we also asked whether SHP-1 plays a role in Treg function. A comparison of the suppressive capabilities of wt and me/me Treg cells demonstrated increased suppressive activity of me/me Treg cells in *in vitro* suppression assays. While this suppression is dependent on stimulation via the TCR, our data indicate that additional factors, which are critical for an Treg function, are regulated by SHP-1. Interestingly when comparing wt and me/me conventional T cells, we observed that SHP-1 deficient CD4+CD25- T cells

are more resistant to Treg-mediated suppression than wt T cells, indicating a role for SHP-1 both in suppression as well as in resistance to suppression. We are currently addressing at the molecular level how SHP-1 regulates these processes.

Conclusions: We expect from these studies to better understand the regulatory role of SHP-1 in conventional and Treg T cells, as well as to gain further insights into the mechanism of suppression by Treg cells. There is increasing evidence that regulatory T cells inhibit the natural as well as therapeutically induced immune response to tumors. Currently, the only way to eliminate regulatory T cells is by targeting CTLA-4 or CD25. Unfortunately, this approach as well removes activated effector T cells since they also express these proteins. Thus, gaining a better molecular understanding of Treg cell development and function might provide new ways of interfering with regulatory T cell activity during tumor immunotherapy.

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P30-6: SIGNALING AND GROWTH INHIBITORY EFFECTS OF DOPAMINE AGONISTS IN BREAST CANCER CELLS

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Dopamine D1 agonist SKF82958 can enhance the transcriptional effects of estrogen receptor (ER) activation while the D1 agonist SKF38393 has been shown to markedly inhibit proliferation of the estrogen receptor-dependent breast cancer-derived MCF-7 cells. Hence, in breast cancer cells dopamine agonists may exert proliferative or anti-proliferative effects by an interaction involving the estrogen and D1-like receptors. Stimulation of dopamine D1-like receptors activates either the adenylyl cyclase (AC) or phospholipase C (PLC) effector pathways. This study was aimed at identifying the specific dopamine receptor/effector system that is responsible for mediating dopaminergic coupling to inhibition of cell proliferation in MCF-7 human breast cancer cells. Here we used dopamine D1 agonists known to activate the AC (SKF85174) or PLC pathways (SKF83959) in GTP binding assays to determine the signaling cascades to which dopamine agonists are coupled in MCF-7 cells. The effects of these agents on [³H]thymidine incorporation was examined in parallel. Our results show that dopamine and dopamine D1 agonists (SKF38393 and SKF83959) stimulate GTP binding in MCF-7 cell membranes while SKF85174 did not. Dopamine and dopamine D1 agonists (SKF38393 and SKF83959) caused a dose-dependent decrease in [³H]thymidine incorporation while SKF85174 was effective only at high concentrations. Both dopamine and SKF38393 were effective in decreasing [³H]thymidine incorporation in MDAMB231 (ER negative) cells. These data suggest a receptor-mediated growth altering effect of dopaminergic agonists that is independent of the estrogen receptor and is differential among dopaminergic agonists.

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P30-7: BLOCKADE OF THE ANGIOTENSIN II SIGNALING PATHWAY DELAYS GROWTH OF BREAST CANCER CELL LINES

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Pleiotrophin (PTN, *Ptn*) is a heparin-binding cytokine expressed in many human breast cancer cell lines; a dominant negative PTN reverses the transformed phenotype of these cells *in vitro* and *in vivo*. Pleiotrophin is expressed in 41/48 human breast cancers tested. In a transgenic mouse breast cancer model, *Ptn* targeted to breast epithelial cells promoted breast cancers cells to a morphological phenotype closely resembling the highly aggressive scirrhous carcinoma in humans, and, induced striking increases in collagen, elastin, and new blood vessels. Pleiotrophin expression was shown earlier to critically regulate expression of key proteins in the renin-angiotensin (AGT) system, suggesting the AGT signaling pathway may be important in breast cancer progression.

Using immunohistochemistry and PCR, AT1, AT2, and ACE were found expressed in human breast cancers. Using PCR, it was found MCF7 (human breast cancer) cells express AT1 and AT2 receptors and AGT and ACE in high levels. MDA-MB-231 (human breast cancer) cells also express AT2, AGT, and ACE. The specific AT1 inhibitor telmisartan significantly delay MCF7 cell growth in monolayer culture compared with untreated cells or cells treated with the AT2 inhibitor PD123,138 or ACE inhibitor captopril. Surprisingly, similar results were obtained with identically treated MDA-MB-231 cells.

The renin-AGT system thus is expressed in human breast cancers. It actively regulates growth of human breast cancer cells *in vitro*. Targeting the renin-AGT system is a potential new treatment for human breast cancers.

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P30-8: A NEW MECHANISM FOR MODULATING THE ACTIVITY OF THE CANCER INVASION PROMOTER CYCLOOXYGENASE-2

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Background and Objectives: Cyclooxygenase-2 (COX2) and its product PGE₂ (a prostaglandin) are known to promote tumor growth by increasing angiogenesis, metastasis, and resistance to apoptosis. Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goals of this proposed research are to unravel a new molecular mechanism for regulating the activity of COX2 and to provide evidence that this mechanism may regulate cell proliferation in cultured breast cancer cells.

Arachidonic acid (AA) is released from the plasma membrane by the action of phospholipase A2 (PLA₂); COX2 then converts AA to prostaglandins. The activity of PLA₂ is known to vary with membrane cholesterol content in a biphasic manner, showing a local minimum at critical sterol mole fractions (C_c) for maximal superlattice formation. We hypothesize that the activity of COX2 will also vary in an alternating manner, allowing plasma membrane cholesterol content to function as a bioswitch to regulate the production of PGE₂. When the cholesterol content is near C_c, the COX2 activity and the PGE₂ production are low. When the cholesterol content deviates significantly from C_c (either >> C_c or << C_c), the COX2 activity, thus the production of PGE₂, becomes high. As such, the cholesterol content near C_c serves as a fine-tuning mechanism to regulate COX2 activity and PGE₂ production, and consequently, cancer cell growth and metastasis.

Description: Using methyl-beta-cyclodextrin (mβCD), we are altering cholesterol levels in very small increments (~1 mol%) in the plasma membranes of MCF-7 cells. After cholesterol depletion, COX2 activity levels can be measured using the COX2 activity kit (Cayman Chemicals). The COX2 activity will be then plotted as a function of cholesterol content relative to total membrane lipids in the plasma membrane. Total membrane lipids will be determined by capillary electrophoresis and mass spectrometry. The extent of cancer cell proliferation will be determined fluorometrically using a CyQUANT assay kit from Molecular Probes on cells with varying cholesterol content alterations. All the experiments will be performed in triplicate.

Results: We are currently assessing the results of preliminary COX2 assays. Using methyl-beta-cyclodextrin (mβCD), we are altering the cholesterol levels in the plasma membranes of MCF-7 cells in very small increments over a wide cholesterol concentration range. After cholesterol depletion, COX2 activity levels can be measured in different cell cultures with varying membrane cholesterol concentrations.

Conclusions: If the hypothesis is correct, then reaching a local minimum in COX2 activity at C_c may be a wise therapeutic goal. This concept could be used to develop a new treatment strategy for the reduction of breast cancer tumor growth and metastasis, which could then be used in combination with existing treatments. The idea is that when the membrane cholesterol level deviates appreciably from those values that produce minimum COX2 activity, cells are more prone to cancer proliferation and invasion due to higher levels of PGE₂ production. In this case, one should manipulate the cholesterol content in cell plasma membrane by locally applying cholesterol lowering or enrichment drugs to the troubled tissues via targeted drug delivery technology.

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P30-9: BREAST TUMOR KINASE ENHANCES EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SIGNALING THROUGH INHIBITION OF CBL-MEDIATED EGFR UBIQUITINATION AND DEGRADATION

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Breast tumor kinase (Brk), a nonreceptor protein tyrosine kinase originally cloned from a metastatic breast tumor, is found in the majority of established human breast cancer cell lines and tumor specimens. Brk interacts with epidermal growth factor (EGF) receptor (EGFR) upon receptor activation, sensitizes mammary epithelial cells to EGF stimulation and promotes EGF-induced cell growth, migration, and invasion, but the molecular mechanism by which this kinase participates in EGFR signaling pathway remains to be elucidated. In this study, we found that experimental elevation of Brk expression leads to an increase in both total and tyrosine-phosphorylated EGFR levels through inhibition of EGFR degradation, measured by ³⁵S-methionine labeling pulse-chase experiments. Further, we found that Brk prevents EGF-induced recruitment of Cbl protein to the fraction of cell membrane, thereby decreasing EGFR-Cbl association and the subsequent ubiquitination of EGFR. Stimulation of cells with EGF enhances EGFR and Brk association whereas treatment of cells with gefitinib (a small molecule EGFR tyrosine kinase inhibitor) decreased their association. Experimental mutation of the Cbl-binding site on the EGFR (Y1045F) abolishes EGF-induced association of EGFR/Brk and phosphorylation of Brk, indicating that Y1045 phosphorylation of EGFR is required for EGF-induced EGFR/Brk association

and Brk phosphorylation. Interestingly, however, the association of Brk with EGFR appears to be SH2 domain-independent because mutation of Brk in its putative SH2 (R105L) or SH3 (W44A, Y66F) domain does not affect the extent of its association with EGFR. On the other hand, experimental elevation of the wild-type Brk, expression of a kinase-dead Brk (K219M) or expression of the constitutively activated Brk-Y447F, particularly the latter, also led to a reciprocal increase in tyrosine phosphorylation on Y1045 (not on other sites including Y845, Y992, Y1068, or Y1172). Moreover, we found that an active Brk fusion protein can phosphorylate EGFR directly on Y1045 in vitro, and that the level of EGFR phosphorylation on Y1045 is decreased when the expression of Brk was knocked down with siRNA. Last, we demonstrated that this EGFR/Brk interaction confers cellular resistance to the anti-EGFR blocking monoclonal cetuximab-induced inhibition of cell proliferation and migration. In summary, our findings revealed a novel function of Brk in prolonging the half life of EGFR through interfering with Cbl-EGFR association at the Y1045 site of EGFR, resulting in persistent activation of EGFR signaling and accordingly cellular resistance to the EGFR-targeted cancer therapy.

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P30-10: TARGETING PROTEIN O-GlcNAc MODIFICATIONS INHIBITS BREAST CANCER PHENOTYPES IN VITRO

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Background and Objective: Compared to normal cells, tumor cells universally upregulate glycolysis, resulting in increased glucose consumption. Although a number of nutrient signaling pathways have been extensively studied in cancer cells, the role of the hexosamine signaling pathway has remained unexplored in tumor cells. The hexosamine signaling pathway converts glucose and glucosamine to UDP-GlcNAc, which acts as a donor for posttranslational modifications of O-linked β-N-acetylglucosamine (O-GlcNAc) to Ser/Thr residues on a diverse set of proteins. The single enzyme, O-GlcNAc transferase (OGT), catalyzes addition of O-GlcNAc to cytosolic and nuclear proteins and can function as a novel dynamic regulatory modification in many ways analogous to phosphorylation. The role of O-GlcNAc modifications as it relates to cancer has not been previously investigated.

Methods: Immortalized normal mammary epithelial cells (MCF-10A, MCF-2A) were compared to breast cancer cell lines (MCF-10A-NeuT, MCF-7, SKBR3, BT20, MDAMB231, MDAMB453, and MDAMB468) for levels of UDP-GlcNAc, levels of O-GlcNAc modified proteins, and OGT levels. MCF-10A cells stably overexpressing the mutant form of ErbB2 (NeuT) and breast cancer cells MDA-MB-231 were treated with RNAi (siRNA and shRNA) against control or OGT sequence. In addition, cells were also treated with specific OGT inhibitor IX. Cells were then analyzed in soft agar assay, three-dimensional (3D) culture, or transwell invasion assay.

Results: We show that breast cancer cells, when compared to normal mammary epithelial cells upregulate the hexosamine signaling pathway including increased UDP-GlcNAc levels, O-GlcNAc modifications, and contain elevated expression of the O-GlcNAc transferase (OGT). Moreover, inhibition of O-GlcNAc modifications by targeting OGT, via RNAi or with a specific inhibitor, leads to regression of tumor phenotypes in vitro including inhibition of colony formation in soft agar, as well as growth and invasion in 3D culture. The inhibition of tumor cell growth by targeting OGT is associated with increased expression of the cell cycle inhibitor p27. Importantly, targeting OGT had no effect in growth and differentiation of normal mammary epithelial cells.

Conclusions: We show, for the first time, an increase in the hexosamine signaling pathway leading to elevated O-GlcNAc modifications in breast cancer cells and that inhibiting this pathway may be a novel therapeutic target for breast cancers.

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P30-11: INHIBITION OF STAT3 ACTIVATION BY RKIP IN BREAST CANCER

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Molecular-targeted therapies are becoming a prominent feature of modern oncology where the therapeutic goal is to trigger tumor cell death (apoptosis). In human breast cancer, the expression of a novel cellular target, Raf kinase inhibitory protein (RKIP), is robustly induced in response to clinically relevant anticancer drugs. The direct correlation of RKIP expression with the extent of apoptosis indicates that the induction of RKIP is one of the mechanisms that sensitizes breast cancer cells to apoptotic signals in response to DNA damage by chemotherapeutic agents. The importance of RKIP in the pathogenesis of breast cancer is supported by findings that RKIP is a clinically relevant metastasis suppressor gene of human breast cancer. RKIP is a negative regulator of the mitogen-activated protein kinase (MAPK) cascade initiated by Raf-1 and nuclear factor kappa B (NF-κB). STAT3 (signal transducers and activators of

transcription) is an oncogene. Intriguingly, STAT3 constitutive phosphorylation is abundant in breast cancer cells and promotes tumor cell survival. Recent findings indicate that STAT3 serine, tyrosine phosphorylation, and acetylation within the C-terminal region is required for STAT3 activation. Given that STAT3 in cells is phosphorylated by MAPK, exploiting RKIP as an activator of apoptosis and simultaneous inhibitor of STAT3 phosphorylation is an unexplored therapeutic approach for the treatment of breast cancer. The goal of this study was to elucidate a new axis connecting RKIP to the STAT3 signaling pathway in human breast cancer. Similar to other tumor cells, the growth of breast cancer cells can be dependent upon the cytokine interleukin-6 (IL-6). IL-6 primarily mediates its effects on breast cancer cells by activating the JAK/STAT pathway. IL-6 also activates the Ras-dependent signaling pathway that includes intermediate steps involving Raf, MEK (MAP kinase kinase), and MAPK. Since RKIP Raf-mediated MAPK activation, we examined the effect of RKIP on IL-6-mediated STAT3 activation. Our results indicate that RKIP inhibits IL-6-mediated STAT3 activation as measured by STAT3 luciferase reporter assay. STAT3 is tyrosine phosphorylated by JAKs and TYK after IL-6 stimulation. To explore the mechanism of the inhibition of STAT3 activation, breast cancer cells were transfected with expression plasmids for JAK1-3 or TYK and RKIP. Our results demonstrate that RKIP inhibited JAK1, 2-mediated STAT3 activation and tyrosine phosphorylation. Using the same experimental approach, we also determined that RKIP significantly inhibited activated Raf- and Src-mediated STAT3 activation and tyrosine phosphorylation. Thus, RKIP behaves as a dual tyrosine kinase inhibitor by blocking JAK and Src kinase activities. We examined the effect of ectopic RKIP expression on Bcl-2 and VEGF, which are STAT3 transcriptional targets, after IL-6 stimulation. IL-6 treatment resulted in increased levels Bcl-2 and VEGF. Ectopic RKIP expression significantly reduced the expression of the anti-apoptotic protein Bcl-2 and the angiogenic factor VEGF. Given the realization of the critical role played by STAT3 in breast cancer progression, identifying molecular mechanisms that antagonize STAT3 activation (in this case via RKIP) is a unique therapeutic approach. The validation of this axis would provide an innovative step in the understanding of breast cancer progression on a molecular scale.

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P30-12: THERAPEUTIC IMPLICATIONS OF OXYGEN-SENSITIVE NOTCH SIGNALING IN BREAST CARCINOMA

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Background and Objectives: Hypoxia affects stem cell function and survival by maintaining cell phenotype, inducing loss of differentiation markers, and blocking differentiation. Notch signaling maintains the undifferentiated state of breast cancer stem cells. Upregulated notch signaling in mouse mammary gland leads to tumor formation suggesting a role for Notch signaling in breast tumorigenesis. SHARP (SMRT/HDAC-1-associated repressor protein) plays an essential role in the Notch signaling pathway. Binding of SHARP to RBP-Jk/CBF-1 results in the repression of RBP-Jk/CBF-1-activated genes. During the activation of Notch signaling, the SHARP protein is replaced by NICD (Notch intracellular domain) resulting in the conversion of the repressor complex to an activator complex. In our analysis, we had identified SHARP and Notch3 as putative hydroxylation targets for prolyl hydroxylases based on the presence of common hydroxylation and pVHL binding motifs [SHARP (LSRLAP³⁶⁴⁴) and Notch3(LLPLAP²¹⁹¹)]. This suggests that both these proteins, SHARP and Notch3, are regulated in a posttranslational manner by hydroxylation and subsequent recruitment of ubiquitin complexes. Thus, the hypothesis is that "Notch signaling is modulated by SHARP and Notch3 during hypoxia." To test this hypothesis, two objectives are proposed.

Specific Aim 1 – To study the interaction of pVHL with SHARP and Notch3 and its role in Notch signaling during differing oxygen levels.

Specific Aim 2 – To study the role of hypoxia-mediated regulation of SHARP and Notch3 in breast cancer stem cell survival and differentiation.

Methodologies: To address Aim 1, breast carcinoma cells including MCF7, MDA231 will be treated with hypoxia and hypoxia mimetic drugs, and the protein levels of SHARP and Notch 3 will be analyzed by western blot. Immunoprecipitation and reporter assays will also be used in this study. For Aim 2, stem cells generated from MCF7, site-directed mutagenesis, cell survival, differentiation-specific markers, and apoptosis assays will be used.

Results: We found that Notch3 levels do not show expected posttranslational regulation in response to hypoxia as well as to several hypoxic mimetic agents (such as desferrioxamine and CoCl₂) and proteasomal inhibitor MG132. However, functional analysis using Notch (Hes1) reporter assay clearly demonstrates that pVHL and FIH-1 (factor inhibiting HIF-1) mediate the trans-repression of Notch targets by SHARP in response to NICD1 and RBP-Jk. Studies are under way to demonstrate if SHARP, pVHL, and/or FIH-1 play any role in the signaling mediated by Notch3. Based on the results obtained using histone deacetylase and histone acetyl transferase inhibitors, a model for recruitment of SHARP/RBPJk complexes and its regulation by hypoxia in breast cancer stem cells is proposed.

Conclusions: Role of Notch signaling in stem cell renewal and the interplay with niche may be an important determinant of cell fate and carcinogenesis. Elucidation of the biology of these pathways may identify new targets for therapy.

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P30-13: A NOVEL MECHANISM FOR ANTI-BREAST CANCER EFFECT OF TUMOR-ASSOCIATED MACROPHAGE MANNOSE RECEPTOR ON ANTI-INFLAMMATORY JAK1, STAT3, SOCS PATHWAYS

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Substantial evidence demonstrates that many cancers including breast cancer arise from a site of chronic inflammation. This strongly suggests that inflammatory cells that exist in the breast cancer microenvironment play a crucial role in cancer pathogenesis and progression. Tumor-associated macrophages (TAMs) are the pivotal members of inflammatory cells within the breast cancer stroma. Upon activation, the TAMs can release a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators that promote breast cancer pathogenesis, growth, and invasion. In this study, we examined whether mannose receptor (MR) could mediate anti-inflammatory mechanisms in breast cancer cells and in TAMs. We observed that MR was expressed in macrophages and was tyrosine phosphorylated at serine/threonine residues. Furthermore, transfection of MR in breast cancer cells resulted in the expression of MR in breast cancer cells and the activation of MR and JAK-1, leading to STAT3 nuclear translocation and upregulation of SOCS-1, 3 protein expression and STAT3 activation in breast cancer cells and TAMs. This pathway of JAK/STAT/SOCS is an important anti-inflammatory pathway. In addition, we observed an increase in the release of anti-inflammatory cytokines such as IL-6, IL-8, TNF- α , and IL-1b in MDA-MB-231 cells transfected with MR. Knockdown of mannose receptor in macrophages and breast cancer cells resulted in significant inhibition of its function in macrophages and blocked IL-8 release in breast cancer cells. Taken together, this study provides important information on MR in tumor-associated macrophages and in breast cancer cells and its roles in breast tumor progression.

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P30-14: REGULATION OF LETHAL GIANT LARVAE IN EPITHELIAL CELLS

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High motility and invasiveness of malignant breast cancer cells depend on their altered polarity and adhesion properties. The proper organization of normal epithelial tissues, including breast epithelium, is regulated by Lgl genes. The Lgl proteins are known to have anticancer properties in the fruit fly and were proposed to decrease the frequency of epithelial cancers in humans. We wanted to establish the causal relationship between the presence and localization of Lgl proteins, cell proliferation, and cell ability to undergo epithelial-mesenchymal transformation. To that end, we developed a tetracycline-inducible system that allows us to trigger Lgl expression in epithelial cell lines and observed Lgl-dependent effects on the proliferative capacity of carcinoma cells in vitro. Carcinoma cells expressing Lgl divided much slower than control cells consistent with the idea that Lgl is a tumor suppressor that inhibits growth of mammalian cancer cells. We also studied the effects of Wnt proteins, known regulators of cell proliferation and cell motility, on Lgl localization in embryonic ectoderm cells of *Xenopus* embryos. Among several tested Wnt ligands, Wnt5, which has been reported to influence cell motility and the cytoskeleton, caused dissociation of Lgl from the cell cortex to the cytoplasm. This effect appeared to be independent of Lgl phosphorylation by atypical protein kinase C, which had been previously reported to influence Lgl localization. These studies may result in new approaches that block metastatic cell behavior by restoring Lgl-dependent epithelial polarity and may lead to the design of physiologically relevant drugs against breast cancer.

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P30-15: DELPHINIDIN: A NOVEL AGENT FOR INHIBITION OF BREAST TUMOR KINASE SIGNALING BY TARGETING EGFR

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Aberrant expression and/or activation of signaling pathways downstream of the epidermal growth factor receptor (EGFR) contribute to progression, invasion, and maintenance of the malignant phenotype in breast cancer. EGFR is expressed at high levels in at least 30% of breast cancers and is associated with poor prognosis. Upon

EGF stimulation, breast tumor kinase (Brk) is recruited to the EGFR, and this event activates the catalytic activity of Brk, which in turn phosphorylates paxillin, a binding partner and substrate for Brk. Currently, many synthetic inhibitors of EGFR are known, but their widespread use is limited because of their unacceptable cytotoxic effects on normal cells. Therefore, identification of a natural, nontoxic agent(s) as an inhibitor of EGFR is of utmost importance. Delphinidin, a major anthocyanidin known to be present in pigmented fruits and vegetables possesses potent antioxidant and antiproliferative properties. In this study, employing EGFR-positive breast cancer AU-565 cells and immortalized MCF-10A cells, we evaluated the effect of delphinidin on EGFR and its downstream signaling pathways. Delphinidin (5–40 mM; 3 h) treatment of both AU-565 cells and MCF-10A cells was found to result in a dose-dependent decrease in the phosphorylation of specific tyrosine residues of EGFR at 1068, 1045, and 845 sites. The signaling pathways induced by activated EGFR include the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPKs), both of which play a significant role in the mitogenic and cell survival responses mediated by EGFR. Therefore, we analyzed the expression of these proteins and found that delphinidin treatment of AU-565 and MCF-10A cells inhibited the (1) activation of PI3K and (2) phosphorylation of AKT and MAPKs in a dose-dependent manner. In additional experiments, serum-starved AU-565 cells were treated with delphinidin (5–40 mM; 3 h) and then incubated without or with EGF (50 ng/ml) for 15 minutes. We found that delphinidin treatment of AU-565 cells inhibited EGF-induced phosphorylation of EGFR, AKT, and MAPK, and activation of PI3K in a dose-dependent manner. Since EGFR is an upstream regulator of Brk signaling pathway through which Brk mediates EGF-induced phosphorylation of paxillin and activation of Akt, and involved in cell migration and invasion, we next determined the effect of delphinidin on Brk signaling pathway mediated through EGFR. We found that treatment of AU-565 cells with delphinidin inhibited EGF-induced (1) protein expression of Brk, (2) phosphorylation of paxillin at Tyr³¹ and Tyr¹¹⁸, (3) activation of Rac 1 protein, and (4) cell invasion. We then compared the growth inhibitory effects of delphinidin (5–40 μ M; 48 h) and found that it resulted in a decrease in cell growth of AU-565 and MCF-10A cells but had only minimal effects on normal mammary epithelial 184A1 cells. Furthermore, treatment of AU-565 cells with delphinidin resulted in (1) induction of apoptosis, (2) cleavage of PARP protein, (3) activation of caspase-3, and (4) downregulation of Bcl-2 with an increase in the expression of Bax. In summary, this study identifies a naturally occurring dietary agent delphinidin as an effective inhibitor of EGFR signaling in breast cancer cells that acts through a novel Brk signaling pathway and holds great promise for its treatment. We suggest that delphinidin could be developed as an agent for the management of EGFR-positive human cancers.

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P30-16: ROLE OF BREAST TUMOR KINASE IN BREAST CANCER

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Breast Tumor Kinase (Brk) is a nonreceptor tyrosine kinase that is expressed in 70% of human breast cancers but is undetectable in normal or benign tissues. The role of Brk in breast cancer development is not known, but it has been demonstrated to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3, activate PI3-kinase (PI3K)/Akt signaling, and promote cell proliferation. Tyrosine kinases regulate wide range of cellular function including cell proliferation and apoptosis and play a crucial role in cancer development. Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR and HER2, are highly expressed in breast cancer and are associated with poor prognosis. The PI3K/Akt pathway mediates EGF-induced cell proliferation and survival and causes cells to become refractory to anticancer drugs by activating the mechanism of anti-apoptosis. Since Brk regulates EGF and PI3K/AKT signalings, it is likely that Brk promotes both cell growth and survival. Moreover, up-regulation of Brk in breast cancer cells may alter cellular sensitivity to therapeutic agents that target to inhibit the activity of EGF receptors. The aims of this study are as follows: first, determine the effect of Brk on the response of breast cancer cells to EGF receptor inhibitors and second, investigate the mechanism underlying the regulation of cell proliferation by Brk. The study was carried out in breast cancer cell lines T47D and MDA231. Ectopic expression of Brk has no significant effect on EGFR/HER2 inhibitor GW2974-induced apoptosis. Similarly, suppression of Brk expression in T47D cells, which expression high levels of Brk, does not enhance cell sensitivity to GW2974. These results suggest that Brk has no direct effect on cell response to the drug. Suppression of Brk gene expression by RNA interference (RNAi) causes a decrease of cell proliferation rate, which is consistent with previous report that Brk induces cell proliferation. This decrease of cell proliferation rate is accompanied with the increased expression of cell cycle inhibitor p27. Treatment of the cells transfected with RNAi targeting to knockdown Brk with PI3K inhibitor LY294002 abolishes the increase of p27 expression. Together, these data suggest that Brk deregulates cell cycle by inhibiting p27 expression, and the mechanism is mediated by PI3K/Akt pathway.

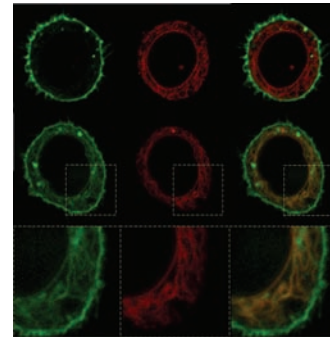
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P30-17: ROLE OF THE Sec61 TRANSLOCON IN EGF RECEPTOR TRAFFICKING TO THE NUCLEUS AND GENE EXPRESSION

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The epidermal growth factor (EGF)-dependent trafficking of the intact EGF receptor to the nucleus and its requirement for growth factor induction of cyclin D and other genes has been reported. Unresolved is the mechanism by which this or other transmembrane proteins are excised from a lipid bilayer before nuclear translocation. Purification of endoplasmic reticulum (ER), ER retrotranslocation assay in vitro and confocal microscopy were employed. We report that, after the addition of EGF, the cell surface EGF receptor is trafficked to the endoplasmic reticulum (figure) where it associates with Sec61 beta, a component of the Sec61 translocon, and is retrotranslocated from the ER to the cytoplasm. Abrogation of Sec61 beta expression prevents EGF-dependent localization of EGF receptors to the nucleus and expression of cyclin D. This indicates that EGF receptors are trafficked from the ER to the nucleus by a novel pathway that involves the Sec61 translocon.



MDA-MB-468 cells were co-transfected with EGFR-mGFP (green) and the endoplasmic reticulum (ER) protein calreticulin-RFP (red). The cells were treated with or without EGF, and live cell images were obtained by confocal microscopy. In the absence of EGF (upper panel), the receptor is predominantly distributed at the plasma membrane. Only a very low level of EGFR-mGFP is detectable in the ER. After incubation with EGF (middle panel), however, there is a large increase in EGFR-mGFP signal that overlaps with the calreticulin-RFP signal. This occurs throughout the lattice-like ER network and includes the nuclear membrane, the outer portion of which is contiguous with ER (lower panel).

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P30-18: THE ROLE OF β -TRCP UBIQUITIN LIGASE RECEPTOR IN THE DEVELOPMENT OF BREAST CANCER

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Beta-transducin repeats-containing proteins (β -TrCP) are the substrate recognition subunits for the SCF ^{β -TrCP} E3 ubiquitin ligases and play a pivotal role in the regulation of cell division and various signal transduction pathways, which, in turn, are essential for many aspects of tumorigenesis. β -TrCP is required for activation of antiapoptotic transcription factor NF- κ B by activated v-Ras and v-RAF, and hence, an elevated expression of β -TrCP may contribute to malignant transformation of mammary epithelial cells. In order to determine whether β -TrCP function is essential in mammary carcinogenesis, we developed double transgenic mice with inducible expression of dominant-negative β -TrCP2 in mammary epithelia. Induction of dominant-negative β -TrCP2 expression in mammary epithelia inhibited activity of endogenous β -TrCP and resulted in reduced nuclear accumulation of p65/RelA member of NF- κ B family of transcription factors. Induction of dominant-negative β -TrCP2 expression in mammary epithelia attenuated DMBA-induced hyperplasia. Overexpression of β -TrCP2 was also observed in human breast cancers, and its activity was found to be essential to maintain antiapoptotic properties of human breast cancer cells.

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P30-19: DIFFERENTIAL REGULATION OF CALCIUM INFLUX IN BREAST CANCER CELL LINES

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Breast cancer is the most common malignancy among women and is the fifth leading cause of cancer mortality. Current understanding of its etiology and progression is limited, and its development appears to be heterogeneous. Intracellular Ca^{2+} signaling is a key regulator of cell cycle progression, proliferation, differentiation, and apoptosis. However, much less is known about the role of aberrant Ca^{2+} signaling in disease, and the mechanisms involved in calcium homeostasis in breast epithelia are poorly characterized.

Transient receptor potential family members of membrane proteins (TRPV5 and TRPV6) constitute the Ca^{2+} influx pathway in epithelia and annexin 2 (anx 2)-S100A10, the first associated proteins to be identified, regulate the translocation and function of these channels in kidney and intestine. Previous studies have shown (1) that TRPV6 is strongly expressed in breast cancer and (2) selective expression of anx 2 in MDA-MB231 cells but not in poorly invasive MCF-7 cells, suggesting participation in invasive breast cancer. Since we have previously shown that cAMP/protein kinase A (PKA) regulates anx 2-S100A10/TRPV6 complex formation in airway and gut epithelia and that this complex regulates calcium uptake in gut epithelia, we speculated that anx 2-S100A10/TRPV6 complex formation and calcium influx may be differentially regulated in breast cancer cells.

We found that despite the presence of all three proteins (anx 2, S100A10, and TRPV6) in breast cancer cells, there was no anx 2-S100A10/TRPV6 complex formation. Furthermore, stimulation of breast cancer cells with cAMP agonists does not induce complex formation. Immunohistochemical analysis of the three proteins shows differential staining in breast cancer tissue. Analysis of calcium influx shows differential PKA-dependent 45Ca^{2+} uptake between two different breast cancer cell lines (invasive and noninvasive). This suggests an important pathological role for the annexin 2-S100A10/TRPV6 complex and highlights its potential as a therapeutic and diagnostic target.

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P30-20: BINDING OF TISSUE INHIBITOR OF METALLOPROTEASES-2 (TIMP-2) TO MEMBRANE-TYPE 1 MATRIX METALLOPROTEASE (MT1-MMP) CONTROLS HUMAN BREAST CARCINOMA GROWTH BY A NON-PROTEOLYTIC MECHANISM

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Background: Matrix metalloproteinases (MMPs) control tumor invasion and metastasis through the degradation of cell membrane and/or extracellular matrix components. Experimental and clinical data have implicated membrane-type 1 MMP (MT1-MMP), a transmembrane MMP with a short cytoplasmic domain and an extracellular catalytic domain, as the most important MMP. High levels of MT1-MMP are associated with aggressive human breast carcinoma and correlate significantly with lymph node and distant metastases, clinical stage, and tumor size. MT1-MMP forms a complex with its physiological protein inhibitor, tissue inhibitor of metalloproteinase-2 (TIMP-2). Because TIMP-2 inhibits MMP activity, elevated TIMP-2 levels would be expected to block tumor progression. Surprisingly, high levels of TIMP-2 in primary human breast carcinomas often correlate with a negative prognosis and are associated with development of distant metastases. Based on these observations, we hypothesized that TIMP-2 binding to MT1-MMP activates intracellular signaling that upregulates human breast cancer cell proliferation and migration through a proteolysis-independent mechanism.

Methods: To test this hypothesis, we used human MCF-7 mammary carcinoma cells transfected with MT1-MMP cDNA under control by the tetracycline-controlled transactivator (Tet-On and Tet-Off). MT1-MMP mutants lacking proteolytic activity or the cytoplasmic tail were also transfected into MCF-7 cells. Activation of intracellular signaling was characterized by western blotting with antibodies to phosphorylated signaling proteins. MCF-7 cells transfected with wild-type or mutant MT1-MMP were injected into the mammary fat pad of female athymic *nu/nu* mice. Tumor growth was monitored by caliper measurements of two perpendicular diameters.

Results: TIMP-2 binding to MT1-MMP induces rapid (5 min) activation of the Ras - Raf - ERK1/2 signaling pathway. This effect is mediated both by proteolytically inert MT1-MMP and by mutant TIMP-2 devoid of MMP inhibitory activity, but not by MT1-MMP devoid of cytoplasmic tail. The signaling effect of TIMP-2 is mediated by interaction of its C-terminal domain with the hemopexin and/or hinge domain of MT1-MMP and a four-amino acid sequence of the cytoplasmic tail. MT1-MMP-

mediated activation of ERK1/2 upregulates cell migration and proliferation in vitro. In vivo, proteolytically inactive MT1-MMP promotes human MCF-7 mammary tumor growth, whereas proteolytically active MT1-MMP devoid of cytoplasmic tail did not have this effect.

Conclusions: Our findings show a novel, non-proteolytic mechanism through which MT1-MMP - TIMP-2 interaction controls human breast carcinoma cell functions. Disruption of TIMP-2 - MT1-MMP interaction by the synthetic MMP inhibitors used in clinical trials requires inhibitor concentrations orders of magnitude higher than those pharmacologically obtainable in vivo. This observation may explain their clinical inefficacy. Inhibitors that effectively prevent TIMP-2 - MT1-MMP interaction are therefore necessary to block the proliferation- and migration-inducing signaling mediated by these proteins. Thus, a detailed understanding of this signaling mechanism can have important implications for the development of novel pharmacological treatments to control breast cancer progression.

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P30-21: TRANSFORMING GROWTH FACTOR- β -INDUCED PLASMINOGEN ACTIVATOR INHIBITOR-1 EXPRESSION REQUIRES COOPERATIVE EPIDERMAL GROWTH FACTOR RECEPTOR AND RhoA SIGNALING

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TGF- β 1 and its target gene encoding plasminogen activator inhibitor-1 (PAI-1) are major regulators of the tumor angiogenic response and important contributors to human breast carcinoma progression and metastasis. The increasing complexity of TGF- β 1 action in the tumor microenvironment and vascular system requires analysis of specific TGF- β 1-initiated signaling events that impact PAI-1 transcription regulation. TGF- β 1-induced PAI-1 expression was completely blocked by inhibition of EGFR activity or adenoviral delivery of a kinase-dead EGFR^{K721A} construct. TGF- β 1-stimulated PAI-1 expression, moreover, was preceded by EGFR phosphorylation on Y845 (a *src* kinase target residue) and required pp60^{c-src} activity. Infection with an adenovirus encoding the EGFR^{Y845F} mutant or transfection with a dominant negative pp60^{c-src} (DN-Src) expression vector effectively decreased TGF- β 1-stimulated, but not PDGF-induced, PAI-1 expression implicating the pp60^{c-src} phosphorylation site EGFR^{Y845} in the inductive response. Consistent with these findings, TGF- β 1 failed to induce PAI-1 synthesis in *src* kinase-deficient (SYF^{-/-}) fibroblasts and reexpression of a wild-type pp60^{c-src} construct in SYF^{-/-} cells rescued the PAI-1 response to TGF- β 1. TGF- β 1-induced EGFR activation, but not SMAD2 activation, moreover, was virtually undetectable in SYF^{-/-} fibroblasts in comparison to wild-type (SYF^{+/+}) counterparts, confirming an upstream signaling role of *src* family kinases in EGFR^{Y845} phosphorylation. Genetic EGFR deficiency or infection with EGFR^{K721A} virtually ablated TGF- β 1-stimulated ERK1/2 activation as well as PAI-1 expression but not SMAD2 phosphorylation. Transient transfection of a dominant negative RhoA (DN-RhoA) expression construct or pretreatment with C3 transferase (a Rho inhibitor) or Y-27632 (an inhibitor of p160ROCK, a downstream effector of Rho) also dramatically attenuated the TGF- β 1-initiated PAI-1 inductive response. In contrast to EGFR pathway blockade, interference with Rho/ROCK signaling effectively inhibited TGF- β 1-mediated SMAD2 phosphorylation and nuclear accumulation. TGF- β 1-stimulated SMAD2 activation, moreover, was not sufficient to induce PAI-1 expression in the absence of EGFR signaling. Thus, two distinct pathways involving the EGFR/p60^{c-src}/MEK-ERK pathway and Rho/ROCK-dependent SMAD2 activation are required for TGF- β 1-induced PAI-1 expression. The identification of such novel interactions between two TGF- β 1-activated signaling networks that specifically impact PAI-1 transcription may provide therapeutically relevant targets to manage the pathophysiology of PAI-1-dependent breast carcinoma invasion and the associated angiogenic response.

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P30-22: PROTEOMIC ANALYSIS OF NONCANONICAL WNT SIGNALING IN BREAST CANCER CELLS

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Wnts are secreted glycoproteins that activate several distinct intracellular signaling pathways, including those important for cell proliferation, differentiation, and polarity. Wnt5a signaling is known to suppress breast tumor formation and is thought to antagonize the canonical Wnt pathway (β -catenin-dependent Wnt signaling) and thereby attenuate its role in cell proliferation. To better understand how Wnt5a signaling suppresses tumorigenesis, we have generated protein interaction data by mass spectrometry for several intracellular members of the Wnt5a signaling cascade including Inversin, Diversin, Vgl2, PTK7, Ryk, and Scribble. Additionally, we have generated lentiviral fluorescent tools to follow localization of Wnt5a transduction

components during cell morphogenesis. Our fluorescent tools allowed us to observe that Scribble and Vgl2 are localized to cell membranes in confluent cells (acini or 2-dimensional culture), but in breast cell migration assays, Scribble and Vgl re-localize to the leading edge of the migrating cells. Intriguingly, loss of function of Scribble prevents migration of MCF-10A or MDA-201 cells and is correlated with defective actin re-organization. In the future, we hope to characterize whether any of the novel members of the Wnt5a pathway interactome might play a functional role in breast tumor suppression in *in vitro* models and whether they may mark cells/tissues that have an altered propensity for transformation or metastasis.

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P30-23: TARGETING BREAST CANCERS FEATURING ACTIVATING MUTATIONS IN PIK3CA

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The lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3) is a critical second messenger in cell signal transduction. The level of PIP3 is tightly regulated by the activities of two opposing enzymes, phosphatidylinositol 3-kinase (PI3K) and Phosphatase and tensin homolog (PTEN), acting as “on/off” switches. We hypothesize that PI3K activity is tolerated within a relatively narrow window in cells—“too much of PIP3 is just as lethal as too little.” The abnormal elevation of PIP3 levels has been frequently found in human cancers bearing somatic activating mutations in the PIK3CA gene or loss of PTEN function. Interestingly, while both PIK3CA and PTEN mutations occur so frequently in cancers, PIK3CA mutations and PTEN loss are almost mutually exclusive. Since the two genes act as “on/off” switches on PI3K signaling, the reciprocal nature of PIK3CA mutations and PTEN inactivation indicate that while either PIK3CA activation or PTEN loss of function results in an elevation of PIP3 sufficient for oncogenesis, the PIK3CA/PTEN double mutants may elevate PIP3 to a lethal level. To test this hypothesis, we have generated a transgenic mouse model carrying a doxycycline-inducible expression of the oncogenic PIK3CA-H1047R transgene. Additionally, an IRES-luciferase expression cassette was cloned downstream of the oncogenic PIK3CA to monitor the transgene expression. By breeding to an MMTV-rTA line, we have generated bitransgenic Tet-PIK3CA-H1047R/MMTV-rTA mice. We found that the induced mammary glands of bitransgenic mice displayed hyperplasias and mammary tumors following doxycycline induction for 6 days and 7 weeks, respectively. While this oncogenic PIK3CA transgenic model is being characterized, we are generating compound mice carrying Tet-PIK3CA-H1047R/PTEN^{lox/lox}/MMTV-rTA-Cre to examine simultaneous activation of PI3K and inactivation of PTEN in an animal breast tumor model. These studies will provide a new perspective on the relationship of the two key oncogene and tumor suppressors, PIK3CA and PTEN, and the signaling pathway under their control in cell regulation and oncogenic transformation, but also a potential novel therapy to all patients plagued with the common tumorigenic mutations.

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P30-24: BREAST TUMOR KINASE AS A COMPLEMENTARY TARGET FOR SENSITIZING BREAST CANCER TO EPIDERMAL GROWTH FACTOR RECEPTOR-TARGETED THERAPY

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Breast tumor kinase (Brk) is a nonreceptor protein tyrosine kinase containing Src kinase homology domain (SH2 and SH3) and is found in approximately two thirds of human breast cancer specimens, including lymph node metastases. Current knowledge on the roles of Brk in breast cancer development and progression and resistance to treatments is very limited. In present study, we found that overexpression of wild-type Brk, or expression of a constitutively active Brk (Y447F) mutant, leads to a marked increase in the levels of activation-specific phosphorylation of mitogen-activated protein kinases (MAPK) p44/42 and p38, particularly the latter, in several breast cancer cell lines (MCF7, SUM149, and SUM102); the levels of activation-specific phosphorylated Akt were also moderately increased. Compared to SUM102neo cells, SUM102 breast cancer cells transfected for high level of the wild-type Brk showed an enhanced response to epidermal growth factor (EGF)-stimulated cell proliferation, suggesting a role of Brk in sensitizing cells to EGF stimulation. We reasoned that an increased sensitivity to EGF stimulation of Brk-overexpressing cells would imply a decreased dependence of the cells on EGFR ligands and thereby a decreased sensitivity to EGFR blockade with monoclonal antibodies. Accordingly, we hypothesized that an overwhelming high level of Brk expression found in breast cancer patients is a leading cause of the poor response of breast cancer cells to EGFR-targeted therapy. To test this hypothesis, we determined the impact of Brk on cellular response to EGFR-targeted therapy by overexpressing Brk in A431 cells, a cell line that is well known for over-expressing EGFR and for being responsive to treatments with the EGFR-blocking monoclonal antibody cetuximab. We found that experimental elevation in the level of wild-type Brk in A431 cells rendered the cells less sensitive to

cetuximab-induced inhibition of MAPK and phosphoinositide-3 kinase (PI3K)/Akt signaling, and conferred cellular resistance to cetuximab, including resistance to its anti-proliferative effect measured by cell number counting and MTT assay, and to its anti-cellular invasion and migration effects measured by Boyden chamber assay and “wound-scratch” healing assay. Moreover, we found that overexpression of Brk or expression of the constitutively active Brk mutant resulted in an increase in the level of phosphorylated paxillin, a multi-domain adaptor protein that interacts with both structural and signaling molecules involving cell invasion and migration, and an increase in the level of the extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) that stimulates peritumoral fibroblasts to produce matrix metalloproteinases, thereby contributing to tumor invasion and metastasis. Taken together, the data suggested that Brk is an important molecular player that might be critical for breast cancer responses to the EGFR-targeted therapies. Our results justify development of novel therapeutic agents by genetic or small molecule inhibitor approaches for targeting Brk for breast cancer treatment.

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P30-25: RAB25 SMALL GTPASE-MEDIATED MOLECULAR MECHANISM IN BREAST CANCER

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A better understanding of the molecular mechanisms that contribute to breast cancer function, development, and progression are essential in exploring novel targets for therapeutic intervention. Recently, the RAB25 gene has been implicated in progression and aggressiveness of breast cancers as increased RAB25 gene expression correlates with a poor prognosis in breast cancer patients. However, the molecular mechanism(s) by which Rab25 protein mediates its functions remains unknown. We proposed to identify Rab25-specific interacting proteins and signaling transduction molecules in breast cancer cells using fluorescent retrovirus-based molecular two hybrid screen (ReMTH) and reverse-phase protein lysate array (RPPA), respectively. ReMTH utilizes the technical advantages of two emerging technologies, the enhanced retroviral mutagen exon trap (ERM) and protein-fragment complementation assays (PCAs). ERM efficiently traps and tags endogenous genes by integration into the host genome, providing a facile approach to trap host proteins without the need to develop cDNA libraries. In PCA, a green fluorescent protein homolog, improved fluorescent protein (IFP), was split into 2 halves—N-terminal half of IFP (IFPN) and C-terminal half of IFP (IFPC). The two separated halves will not reconstitute or fluoresce spontaneously. When each half of GFP is fused to one of a pair of interacting protein partners, the subsequent protein interaction can restore fluorescence. In an attempt to isolate Rab25-interacting molecules in breast cancer cells, an MCF-7 Tet-off IFPN-Rab25 cell was established, infected with ReMTH-IFPC1 virus, and selected with puromycin. Multiple fluorescence cells were selected. Up to date, we have identified 5 putative Rab25-interacting molecules from the screening. To examine the Rab25-mediated signaling, RPPA was used to profile a panel of signaling transduction molecules, in RAB25 overexpressing or pcDNA transfected control MDA-MB-231 cells, after stimulation with insulin growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-beta (TGFβ), and fetal bovine serum (FBS) for 0.1h (6 min), 0.5h (30 min), 1h, 2 h, 6h, 8h, and 24h prior to total cellular protein isolation. Hierarchical clustering of the data has demonstrated characterized effects on AKT pathway activation by EGF and IGF. In addition, expression of Rab25 prolonged and enhanced the AKT signal transduction at 24h time point. Despite lower in magnitude, FBS (0.5h) and TGFβ (0.1 and 0.5h) treatment resulted in increased and prolonged (24h) AKT activation in Rab25 table expressing MDA-MB-231 cell. We have been successful in utilizing these technologies to identify (1) putative Rab25-interacting molecules and (2) differential activation of signaling pathway by Rab25 in the presence of insulin growth factor, epidermal growth factor, transforming growth factor-beta, and fetal bovine serum. Further studies will be carried out to confirm the Rab25-interacting molecules and the Rab25-mediated signal transduction. Once these events are elucidated, Rab GTPases and their interacting proteins or signaling pathways in which they operate will present novel, unexplored potential targets for therapeutic intervention.

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P30-26: OVEREXPRESSION OF RhoA INDUCES PRENEOPLASTIC TRANSFORMATION OF PRIMARY MAMMARY EPITHELIAL CELLS

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Rho family small GTPases serve as molecular switches involved in the regulation of diverse cellular functions including various cytoskeleton-related events, gene transcription, cell proliferation, and oncogenic transformation of immortal cells. Impor-

tantly, Rho overexpression is frequently seen in many carcinomas. However, most of the published studies utilized immortal or tumorigenic cell lines and there is no publication that examined the ability of Rho small GTPase to overcome senescence checkpoints and induce preneoplastic transformation of human epithelial cells. We demonstrate here, that ectopic expression of wild-type RhoA as well as constitutively active RhoA mutant (G14V) in two independent normal mammary epithelial cells led to their immortalization and preneoplastic transformation. These cells continue to grow for more than 300 population doublings with no signs of senescence, whereas cells that received vector or dominant-negative RhoA mutant (T19N) senesced after 20 population doublings. Significantly, a mutant RhoA-T37A that is known to be incapable of interacting with well-known Rho-effectors, Rho-kinase, PKN, and mDia 1 and 2 was also capable of immortalizing normal mammary epithelial cells. There was a gradual increase in telomerase activity with decline in telomere length in Rho expressing cells, indicating that Rho immortalizes cells by telomerase pathway. Importantly, similar to parental normal cells, Rho-immortal cells have wild-type p53 and intact G1 cell cycle arrest upon adriamycin treatment. Rho-immortal cells were anchorage-dependent and were unable to form tumors when implanted in nude mice. Significantly, in contrast to normal parental cells that formed regular acinus structures, Rho immortal cells exhibited irregular branching morphogenesis when cultured in 3-dimensional cultures. Lastly, microarray analyses of normal and Rho-immortal cells showed changes in several genes that are known to be associated with immortalization and breast cancer progression. Taken together, these results demonstrate that Rho induces pre-neoplastic transformation of normal mammary epithelial cells and this process does not involve Rho-kinase, PKN, and mDia, well-known downstream regulators of Rho signaling pathway.

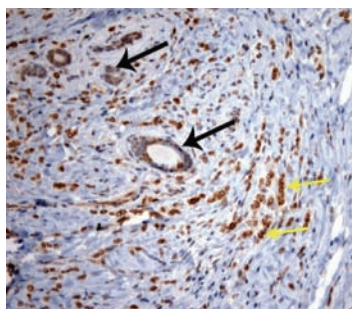
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0231.

P30-27: MECHANISM OF AKT-DEPENDENT JNK INHIBITION: IMPLICATIONS FOR THE PROGNOSIS AND TREATMENT OF BREAST CANCER

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The critical role of the PTEN/PI3K/AKT pathway in the pathogenesis of breast cancer is illustrated by findings of pathologically increased AKT activity in primary tumors. Cell-based studies show that increased AKT activity leads to apoptosis resistance of breast cancer cells. AKT-dependent anti-apoptosis in breast cancer cells, in turn, requires JNK inhibition. Within this framework, we have investigated molecular mechanisms that couple AKT to JNK signaling and may be relevant for the resistance of breast cancer cells to chemotherapeutic intervention. One such mechanism in breast cancer cells is mediated by AKT-dependent inhibition of ASK1. To verify the importance of AKT-dependent ASK1 regulation in human tumors, we have correlated AKT-dependent ASK1 phosphorylation with clinical response and outcome using well-documented breast tumor tissue



ASK1 Hyperphosphorylation in
Lobular Breast Cancer

arrays. In addition to AKT-dependent JNK inhibition through ASK1 phosphorylation, we find that AKT can block JNK signaling in breast cancer cells by regulating the turnover of specific JNK pathway components. This novel mechanism of JNK inhibition requires AKT phosphorylation but not its catalytic activity. The relative importance of substrate phosphorylation versus non-enzymatic scaffolding functions of AKT will be discussed in view of possible molecular targets.

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P30-28: Met RECEPTOR TYROSINE KINASE SIGNALING AND CELL DISPERSAL REQUIRES DORSAL RUFFLE FORMATION REGULATED BY THE Gab1 SCAFFOLD PROTEIN

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The Met receptor tyrosine kinase (RTK) and its ligand, hepatocyte growth factor (HGF) are deregulated in human breast cancer through overexpression, point mutations, and co-expression of the ligand and receptor. Using human breast cancer cell lines in culture, we have shown that processes that correlate with enhanced tumor growth and invasion such as dissociation, motility, and invasion are induced by HGF. Cell dissociation and motility downstream from activated RTKs require changes in the actin cytoskeletal network that occur rapidly through alterations in the plasma membrane, such as the formation of lamellipodia and dorsal ruffles. HGF has been shown to induce dorsal ruffles, which are actin-rich membrane protrusions. However, little is understood about the role of these structures and how they impact on RTK signalling and regulation. Here we show that stimulation of Madin-Darby canine kidney (MDCK) cells with HGF results in the formation of dorsal ruffles that contain both the Met receptor and the scaffold protein Gab1. Gab1 is rapidly phosphorylated and recruited to the Met receptor upon HGF stimulation and is responsible for propagating Met signalling to the PI3K, MAPK, and Crk dependent pathways. We have previously shown that overexpression of Gab1 in MDCK cells induces a sustained activation of the MAPK pathway downstream from the Met receptor. In our current study, we demonstrate that Gab1 enhances the formation of dorsal ruffles in MDCK cells, which are enriched with activated Met receptor and Erk1/2. Importantly we show that overexpression of Gab1 can promote dorsal ruffle formation in HeLa cells that do not normally support these structures. Loss of the Gab1 protein by siRNA severely reduces the number of dorsal ruffles formed in MDCK cells and ablation of these structures using the stilbene drug SITS, delays Gab1 phosphorylation, inhibits MAPK pathway activation and blocks cell dispersal induced by HGF. Confocal microscopy analysis of the Met receptor in both MDCK and HeLa cells shows that the receptor accumulates on dorsal ruffles and internalizes from these sites.

Our data provide a novel role for Gab1 in promoting dorsal ruffle formation downstream from the Met receptor and demonstrate that these structures act as transient signalling compartments. We also demonstrate that proper formation of dorsal ruffles is required to initiate MAPK pathway activation and for biological activities such as cell dispersal and migration downstream from HGF.

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TUMOR SUPPRESSOR GENES I

Poster Session P31

P31-1: PROMOTER HYPERMETHYLATION MEDIATED TRANSCRIPTIONAL SILENCING OF TP73 IS AN IMMORTALIZED EVENT IN THE BREAST CANCER PROGRESSION CONTINUUM IN THE MCF10 MODEL

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Aberrant DNA methylation has been shown to have a causative role, rather than a bystander effect, in the development of cancer. Promoter hypermethylation-mediated inhibition of gene expression is now widely recognized as an important mechanism for the inactivation of growth regulatory genes in the process of malignant transformation. We examined the promoter methylation status of the 22 cancer genes along a continuum of immortalization, transformation, and malignant progression of breast cancer using the MCF10 model. MCF10 cells in a series included initial immortalized untransformed outgrowths (MCF-F and MCF10A) and 5 transformed cell lines recapitulating various stages of benign proliferation (MCF10-AT1, MCF10AT1kcl2), carcinoma in situ (MCF10CA1h cl13), and invasive carcinoma (MCF10CA1d cl1, MCF10CA1a cl1). A panel of 41 gene probes, designed to interrogate 36 unique genes with known associations to breast cancer, was interrogated for alterations in gene copy number and aberrant methylation status (22 genes) using a modification of the multiplex-ligation specific polymerase assay (MLPA) and confirmed using methylation specific PCR (MSP). Of the six aberrantly methylated genes, *TIMP3*, *TP73*, *CDH13*, *IGSF4*, *RASSF1*, and *DAPK*, hypermethylation of *TP73* was observed in all 7 cell lines, implicating *TP73* as a sole epigenetic marker of immortalization. Hypermethylation of *TIMP3*, *RASSF1*, *CDH13*, and *IGSF4* were consistently hypermethylated in all the remaining 5 transformed cell lines. Aberrant methylation of *DAPK1* was first observed in the transformed cell line MCF10-AT1, and reappeared only in the carcinoma cell lines. There was homozygous loss of both the *CDKN2A* and *CDKN2B* gene loci, obviating gene silencing of p14, p16, and p15. Immortalized cell lines MCF-10F and MCF-10A had aberrant methylation of only *TP73* marking this gene as an early epigenetic target of immortalization. Progression events of transformation and malignancy added additional gene silencing targets to include *TIMP3*, *RASSF1*, *CDH13*, and *DAPK1*. In addition to genetic alterations of gains and losses, epigenetic events appear to further undermine a destabilized genomic repertoire along a molecular continuum of breast cancer progression.

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P31-2: A CHEMICAL STRATEGY TO TRAP AND IDENTIFY PROTEINS ASSOCIATED WITH DNMTs

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DNA methylation catalyzed by DNA methyltransferases (DNMTs) results in the covalent addition of a methyl group to the 5 position of cytosine to give 5-methylcytosine. This modification has a profound effect on DNA structure. Promoters that are highly methylated are tightly condensed and cannot support transcription. Hypermethylation of many genes contributes to growth of breast cancers, including the genes encoding the estrogen and progesterone receptors, E-cadherin, and *BRCA1*. Despite the considerable interest in defining which genes are hypermethylated with breast tumors, little is understood about the factors that determine which genes become hypermethylated. With the support from the DOD Breast Cancer Research Program we initiated a new program to develop covalent trapping reagent that can trap DNMTs on DNA more efficiently. We envision that the covalently linked DNMT-DNA complexes can be used as the bait to identify additional protein factors that may interact with the complexes at the specific DNA sequences.

We first developed a set of diazirine-based DNA base modifications. Diazirine phosphore was introduced into either the major or minor groove of DNA as a photocross-linking group via a convertible nucleoside methodology. The resulting DNA probes efficiently cross-linked with DNA binding proteins. The strategy can be readily applied to map out protein-DNA interactions and utilized in proteomic studies to trap and identify proteins from cell extracts. Next, we started developing a disulfide-based DNA modification in hope to trap DNMTs with much higher efficiency than base modifications reported previously. Our efforts to fine tune these new tools and use them to trap DNMTs from cell extracts will be presented. In the next stage we hope to apply these tools to study promoters hypermethylated in breast cancer cells to establish a proteomic framework in which to understand the complex epigenetic changes seen in breast cancer.

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P31-3: Pten TUMOR SUPPRESSOR FUNCTION IS CRITICAL IN THE TUMOR MICROENVIRONMENT

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Objectives of the Study: Stromal fibroblasts are a major component of the tumor microenvironment (TME). In addition to their roles in extracellular (ECM) remodeling and tumor angiogenesis, fibroblasts can act as local and specialized modulators of immune responses, a "stromal address code" for inflammation and cancer. Despite the documented role of the tumor stroma, relatively little is known about the genes mediating the cellular cross-talk between the tumor epithelial and the fibroblasts and immune and endothelial cells in the TME. Pten (phosphatase and tensin homolog) is essential for embryonic development and a tumor suppressor commonly inactivated in many human cancers.

We developed novel genetic tools to target deletion of Pten in stromal fibroblasts, in combination with mouse models of breast cancer, MMTV-Neu and MMTV-PyMT, to investigate the role of PTEN in the tumor microenvironment. When studied in the context of the transgenic MMTV-neu model, Pten deletion in stromal fibroblasts induced rapid and aggressive tumor growth compared to controls. Additionally, Pten ablation in fibroblasts also significantly increased tumor growth when tumor cells were introduced into the mammary fat pad of mice with Pten-null fibroblasts versus wild type. Histopathological examination revealed that Pten inactivation in mammary stromal fibroblasts induced expansion of tumor stroma and ECM, creating a tumor stroma that was remarkably similar to the stroma surrounding human breast tumors. To begin to identify the molecular mechanisms responsible for the Pten fibroblast-specific phenotype, global gene expression in primary mammary fibroblasts was determined. We identified 133 genes upregulated and 25 genes downregulated >4-fold in the Pten null breast fibroblasts versus control. The Pten responsive genes were shown to be involved in inflammation (61%), ECM remodeling—wound healing (23%), and other classes (16%). The biological significance of the microarray results was validated by investigating mammary stroma for the presence of macrophages (F4/80 staining), ECM deposition (Masson's Trichrome and Collagen I), and ECM remodeling (Mmp-9 in situ enzymatic activity). Mammary glands and tumors with Pten-null fibroblasts had massive macrophage infiltration, extensive fibrosis, and increased Mmp-9, confirming the gene expression pattern. Microarray analysis also revealed a ~3-fold decrease in expression of Ets2 in normal mammary gland with Pten present, a result that was confirmed at the protein level. Importantly, conditional deletion of Ets2 in stromal fibroblasts of the mammary gland resulted in a significant decrease in MMTV-PyMT tumorigenesis. Tumors with Ets2-null fibroblasts had reduced ECM deposition and reduced Mmp-9 activity.

Significance: These in vivo data suggest that Pten action in stromal fibroblasts suppresses tumor growth by the regulating interactions between fibroblasts and immune cells and also by affecting ECM remodeling. Ets2 is one target for Pten action, and activation of Ets2 enhances the potential of fibroblasts to promote tumor growth. We identified the Pten-Ets2 axis as a key regulatory component of mammary fibroblasts that can impact tumor development. The Pten mouse model is the first to closely model the human breast cancer stroma and will be useful for mechanistic studies to uncover the role of fibroblasts in tumor progression.

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P31-4: THE ROLE OF FKHR IN MAMMARY GLAND DEVELOPMENT, INVOLUTION, AND TUMORIGENESIS

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Breast cancer growth is regulated by both estrogen and growth factors, and there is considerable cross-talk among these pathways. FKHR, an important mediator of apoptosis, is inactivated by growth factor signaling via PI3 kinase, resulting in enhanced cell survival. We have found that overexpression of FKHR also represses the transcriptional effects of estrogen receptor α (ER). Therefore, FKHR overexpression may inhibit breast cancer cell growth through its dual negative regulatory functions, while loss of FKHR function may lead to ER hyperactivity and increased growth. In this study we further investigated the role of FKHR in mammary gland development, involution, and tumorigenesis.

To generate the conditional mammary-specific FKHR knock-out mouse model, we crossed floxed FKHR with MMTV/Cre mice. FKHR knock-out (KO) in the mammary gland was confirmed by PCR. Mutant mammary tissues in 6-week-old virgin mice displayed a significant increase in the number of ductal ends (331.9 ± 27.3), branch points (262.3 ± 19.8), and the percentage of fat pad filling ($56.8\% \pm 2.6$) as compared to those of wild-type mammary tissues (169 ± 18.2 , 136.3 ± 16.6 , $44.2\% \pm 3.9$, $p < 0.01$, respectively). Mutant mammary tissues also showed excessive budding/feathery-like side branches on the mammary trunks. Some alveolar buds displayed a hyperplastic phenotype. Deletion of FKHR resulted in significantly

increased proliferation in duct end and duct epithelium of the mammary glands. Numbers of cells positive for Ki67 were significantly higher in FKHR deleted mammary tissues (59.5 ± 1.9 vs. 39.5 ± 1.4 , $p < 0.001$). Forced involution study showed that loss of FKHR resulted in reduced apoptosis and delayed involution. Cleaved caspase 3 levels (expressed as the percentage of positive caspase 3 in total cell numbers) decreased in FKHR knock-out mammary glands as compared to those of wild-type controls at day 3 of involution (28.3 ± 2.5 vs. 19.1 ± 2.3 , $p < 0.05$). On day 14 of involution, there were high numbers of cells positive for caspase 3 in FKHR knock-out mammary glands. Thus, FKHR abolition has profound effects on mammary gland normal development and involution, perhaps due to the proapoptotic and ER-regulated functioning of this protein.

The effect of FKHR abolition on tumorigenesis is currently under evaluation. Concluded studies with multiparous FKHR knock-out mice did not suggest an increase in the incidence of mammary tumorigenesis. To evaluate the potential of FKHR elimination in accelerating tumorigenesis that is driven by other specific oncogenes, we crossed floxed FKHR/MMTV/Cre with MMTV/Myc mice. Deletion of FKHR did not accelerate tumor development in Myc transgenic mice. Molecular and histological analysis of tumor nature showed that tumors from bigenic FKHR KO/Myc mice had a higher percentage of ER and Ki67 positive cells as compared to tumors from the Myc alone transgenic line. More tumors from bigenic mice also showed papillary morphology, whereas most tumors from the Myc alone were adenocarcinomas. These results suggest that loss of FKHR promotes cell proliferation but not the development of mammary tumors. Studies to investigate the role of FKHR in promoting tumorigenesis driven by other oncogenes are ongoing.

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P31-5: SCANNING THE EXPRESSION OF THE GENES ON THE P-ARM OF CHROMOSOME 8 AND THE q21-qter REGION OF CHROMOSOME 18 IN BREAST CANCER BY THE SYSTEMATIC MULTIPLEX REVERSE TRANSCRIPTION-PCR (SM RT-PCR) AND DNA MICROARRAY HYBRIDIZATION METHODS

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Burnham Institute

The activation of oncogenes and the inactivation of tumor suppressor genes both play an important role in carcinogenesis. Most changes in these activating/inactivating processes occur in copy number, gene expression, and/or nucleotide/amino acid sequences. Therefore, the determination of copy number and gene expression, together with nucleotide sequencing, helps in the successful identification of oncogenes and tumor suppressor genes. A monoallelic dominant change is typically sufficient for the activation of an oncogene to occur. On the other hand, for the inactivation of a tumor suppressor gene haplo-insufficiency is rare, and the disruption of both alleles is generally necessary. Using the comparative genomic hybridization method that was derived from the two-color fluorescence in situ hybridization of chromosomal spreads and later applied to DNA microarrays, copy number was quantitatively analyzed and numerous maps of chromosomal alterations in cancer were produced. This revealed a significant degree of heterogeneity among a variety of tumors, as well as heterogeneity within the same type of tumor. In breast cancer, frequent gains in chromosomal arms 1q, 3q, 8q, 16p, 17q, and 20q and losses in 1p, 6q, 8p, 13q, 16q, 17p, 18q, 22q, and X, which are indicative of the presence of oncogenes and tumor suppressor genes, respectively, were reported.

Aiming to identify novel genes with tumor suppressor activity, we launched the process of gene expression analysis. We chose the p-arm of chromosome 8 and the q21-qter region of chromosome 18 because in breast cancer they are decreased in copy number most often, which strongly suggests that the region may harbor tumor suppressor genes involved in the pathogenesis of breast cancer. Using the semi-quantitative (but more sensitive) SM RT-PCR method that we developed, and the high-throughput DNA microarray hybridization method, we examined the expression of 273 genes located on the p-arm of the chromosome 8 and 127 genes located on the 18q21-qter chromosomal region in human breast cancer cell lines. For the genes on the 18q21-ter region, we observed partial or entire loss of expression in CCB1, CCDC11, CD226, NP_115536.1, NP_689683.2, RNF152, SERPINB8, and TCF4. An increase in gene expression was atypical for the genes in this region but was established nonetheless with the transcription factor ONECUT2 gene in every one of the cancer cell lines examined. Real-time qRT-PCR experiments confirmed these SM RT-PCR results. Further analysis of breast cancer specimens using real-time qRT-PCR helped verify that the gene expression of CCB1, TCF4, NP_115536.1, and NP_689683.2 was down regulated in the majority of clinical cases of breast cancer. For the genes on the 8p-arm, we observed frequent decreases in expression of two dozen genes and increases in expression of quite a few genes as well. These alterations in gene expression of the cell lines were later confirmed by real-time qRT-PCR, and a number of them were also observed in clinical breast cancer cases. This included down-regulation of the MYOM2, NP_859074, NP_001034551, NRG1, PHYIP

(PHYHIP), Q7Z2R7, SFRP1, and SOX7 genes and up-regulation of the ESCO2, NP_115712 (GINS4), Q6P464, and TOPK (PBK) genes. If the ongoing assessment confirms functional significance of any of these candidate genes, the changes in expression and copy number may become useful and novel biomarkers of breast cancer.

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P31-6: ROLE OF TUMOR METASTASIS SUPPRESSOR GENE, NDRG1, IN BREAST CANCER PROGRESSION

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Background and Objective: Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women in the United States. Unfortunately, a large fraction of patients already have a metastatic disease at the time of a visit to the clinic. Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies "cure" the metastatic disease. Therefore, there is an urgent need for developing a novel therapeutic approach that directly attacks the metastatic tumor cells. The hallmark of malignant cells is a gain of metastatic ability that is controlled by a variety of positive and negative factors. Emerging evidence of a recent study indicates that tumor metastases suppressor gene NDRG1 plays a crucial role in metastatic development of breast cancer cell. Therefore, understanding the molecular mechanism of NDRG1 function is of paramount interest and is expected to significantly contribute to the development of new therapeutic methodology of breast cancer. The objectives of this research are to examine (i) whether NDRG1 functions as a tumor metastasis suppressor in breast cancer and (ii) whether loss of PTEN function down-regulates the NDRG1 gene that leads to metastases.

Methodologies: Eighty-five surgically resected breast tumor specimens were randomly selected from pathology archives of the Akita Red Cross Hospital. The specimens dated from 1993 to 1997 and the ages of patients ranged from 29 to 79 with a mean of 54 years. Complete 5-year follow-up data were available for these patients. To examine the relevance of NDRG1 expression in clinical setting, immunohistochemical analyses were performed on these samples using antibodies to NDRG1 and PTEN, p53 and ER. The Kaplan-Meier method was used to calculate the survival rates, and prognostic significance was evaluated by the log-rank test.

Results: We found that the expression of the NDRG1 protein was significantly reduced in breast tumor cells, particularly in patients with lymph node or bone metastasis as compared to those with localized breast cancer. NDRG1 expression also exhibited significant inverse correlation with the disease-free survival rate of patients and emerged as an independent prognostic factor. We also found that ectopic expression of the NDRG1 suppressed the invasiveness of breast cancer cells in vitro and this suppression was also achieved by treatment of cells with 5-Azacytidine. Furthermore, our results indicate that ectopic expression of PTEN significantly augments the endogenous expression of NDRG1 protein whereas inhibition of PTEN by siRNA decreased NDRG1. Our immunohistochemical analysis also revealed that PTEN expression correlates significantly with NDRG1 in breast cancer patients and that a combination of the two markers, PTEN and NDRG1, emerged as a significantly better predictor of breast cancer patients' survival than either marker alone.

Conclusions: Our results clearly indicate that NDRG1 plays a critical role in suppressing the metastatic advancement of breast cancer and that a combination of PTEN and NDRG1 serves as an informative biomarker to predict outcome of breast cancer patients. Our results also suggest that perturbation of PTEN-NDRG1 pathway is a potential therapeutic option for breast cancer.

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P31-7: REGULATION OF HTERT EXPRESSION AND FUNCTION IN NEWLY IMMORTALIZED p53(+) HUMAN MAMMARY EPITHELIAL CELL LINES

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Immortality is a prerequisite for the accumulation of multiple errors in a single cell lineage during carcinogenesis, and is associated with the reactivation of telomerase activity, which maintains telomeric ends. The requirement of carcinoma cells for telomerase makes it an attractive target for clinical intervention. However, the mechanisms regulating telomerase reactivation and immortalization in human mammary epithelial cells (HMEC) during carcinogenesis are largely unknown. This project used an immortalized HMEC line developed in our lab, 184A1, to study telomerase

reactivation and telomere protection in newly immortalized p53(+) HMEC. Prior work indicated that p53 can suppress most, but not all, telomerase expression in early passage 184A1, leading to ongoing gradual telomere erosion. When telomeres became extremely short (mean TRF <3 kb), most growth stopped, and levels of the p53-dependent cyclin-dependent kinase inhibitor p57 KIP2 became elevated. A mechanistically undefined process termed conversion then gradually relieved the p53-mediated repression of hTERT leading to full telomerase reactivation, stabilization of telomere length, resumption of good growth, and a gradual decrease in p57 levels. Surprisingly, although telomere lengths were exceedingly short and would be expected to lead to telomeric associations and mitotic failure, there was no evidence of widespread genomic instability before or during conversion. We hypothesized that p57 might prevent genomic instability by inhibiting growth until there was sufficient telomerase to protect the telomeric ends. Therefore, our aims were to test whether the low telomerase activity, along with the elevated p57 expression, suppressed genomic instability, whether inhibition of telomerase activity and/or p57 function might efficiently kill the newly immortal cells, and the role of p53 in telomerase regulation of early184A1. Our studies have shown the following: (1) Inhibition of telomerase yielded accelerated growth arrest and p57 elevation compared to controls, and a failure to become fully immortal. No evidence of p53 activation or a DNA damage response was seen. These data suggest that the low telomerase levels in early 184A1 can maintain the shortest telomeric ends until conversion, and that p57 can elicit growth arrest in response to short, but still protected telomeres. (2) Similar to telomerase inhibition, stable reduction of p57 expression in early, but not fully immortal 184A1, using shRNA or a dominant-negative p57 mutant, produced rapid growth arrest. This arrest was associated with evidence of a DNA damage response, including activation of p53 and up-regulation of p21CIP1. Additionally, conversion to full telomerase reactivation was prevented. This latter result was unexpected, leading us to now hypothesize that growth inhibition from elevated p57 may indeed protect the p53(+) pre-conversion HMEC lines from genomic instability; however, expression of p57 is also required to alleviate the p53-mediated telomerase suppression. In the absence of p57, DNA damage signals can activate p53 to elicit growth arrest, similar to what we observed in senescent finite lifespan p53(+) HMEC with critically shortened telomeres. Altogether, our data support the possibility that newly immortal p53(+) HMEC, possessing low telomerase activity and very short telomeres, may be especially vulnerable to therapies that target telomerase and/or propel cells into catastrophic genomic instability.

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P31-8: MODULATION OF PTEN ACTIVITY IN HUMAN BREAST CANCER

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Background and Objectives: PTEN tumor suppressor is inactivated in multiple human cancers. Mutation in PTEN is the cause of Cowden's disease syndrome with carriers predisposed to breast cancer. Furthermore, PTEN expression is reduced with the progression of sporadic breast cancer. The regulation of PTEN intrinsic biochemical properties has not been fully elucidated. The aim of this project is to decipher the role of post-translational modifications in regulating PTEN biochemical properties in breast cancer.

Methodologies: The role of phosphorylation in regulating PTEN membrane-targeting and catalytic functions was characterized by using a panel of site-directed mutants. The existence of aberrant post-translational modifications in a panel of human breast cancer was investigated.

Results: Characterization of a panel of PTEN phosphorylation site mutants revealed that mutating Ser385 to alanine (S385A) promoted membrane localization in vivo and phosphatase activity in vitro. Furthermore, S385A mutation was associated with a substantial reduction in the phosphorylation of the Ser380/Thr382/Thr383 cluster. We further identified an interaction between the 71-amino acid carboxyl-terminal tail region and the CBR1 motif of the C2 domain, which has been implicated in membrane binding. In addition, a synthetic phospho-mimic peptide encompassing the phosphorylation site cluster between amino acid 368-390 within the tail region mediated the suppression of PTEN catalytic activity in vitro.

By examining a panel of breast tumor cell lines, we have identified a metastatic breast carcinoma line with a PTEN species displaying aberrant mobility on a SDS-PAGE gel. Nucleotide sequencing analysis has identified a single point mutation in exon 8 of the PTEN gene. This genetic modification appears to alter the post-translational processing of PTEN.

Conclusions: The phosphorylation of Ser385 is necessary for priming phosphorylation events in PTEN carboxyl-terminus. Dephosphorylation at this site stimulates PTEN membrane-binding and catalytic activities. The kinase(s) and phosphatase(s) responsible, if identified, will be valuable therapeutic targets for breast cancer treatment.

The discovery of a novel PTEN mutation in a metastatic breast cancer may reveal novel post-translational events. The further characterization of the underlying mechanisms may lead to new drug targets that could enhance PTEN expression in breast cancer cells.

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P31-9: NOVEL BINDING INTERACTIONS OF THE HUMAN LYSYL OXIDASE PRO-PEPTIDE REGION

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Lysyl oxidase is an extracellular enzyme critical for the normal biosynthesis of collagens and elastin. Lysyl oxidase is synthesized and secreted as an inactive 50 kDa pro-enzyme (Pro-LOX), which is processed by proteolytic cleavage into a functional 30 kDa enzyme (LOX) and an 18 kDa pro-peptide (LOX-PP). Lysyl oxidase gene expression was found to inhibit the transforming activity of *ras* in NIH 3T3 fibroblasts and the gene was hence named the "*ras* reversion gene" (*rrg*). Lysyl oxidase is down-regulated in *ras*-transformed cells and in many cancer cell lines.^{1,2} Moreover, aberrant expression of lysyl oxidase or inactivation by loss of heterozygosity is observed in many human cancers such as gastric cancer.³ Recently, our group demonstrated that the pro-peptide region of LOX mediates the *ras* reversion activity of the LOX gene in NIH 3T3 cells,² in breast cancer cells driven by the Her2/neu receptor⁴ and in human lung and pancreatic cancer cells.⁵ Specifically, LOX-PP was found to be a potent inhibitor of the epithelial-to-mesenchymal transition (EMT) of breast, lung, and pancreatic cancer cells in vitro^{4,5} and tumor formation in vivo.⁴ To investigate the molecular mechanisms of the tumor suppressor activity of the LOX-PP, we performed a yeast two-hybrid assay with a human mammary gland cDNA library to identify novel LOX-PP interacting proteins. Five hundred and seventy five clones were identified in the primary screen. Library plasmids from the clones that were confirmed in a secondary screen for β -galactosidase expression were isolated and identified by DNA sequence analysis. Several candidate factors have been identified, including a phosphatase and a novel glycoprotein of the extracellular matrix. Work is in progress to test their roles in the tumor suppressor activity of the LOX-PP.

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P31-10: MATRIX STIFFNESS REGULATES HoxA9 MODULATION OF BREAST TUMOR PHENOTYPE

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The extracellular matrix and its receptors, the integrins, modulate development and maintain tissue homeostasis through a network of soluble and insoluble cues. Alterations in integrins have been strongly implicated in both tumorigenesis and metastasis. The identification of upstream regulators of integrin expression and activity may provide a better marker for predicting adhesion-dependent breast tumor behavior. The homeobox family of genes, which plays a critical role in tissue development, can influence integrin and ECM expression, and is frequently altered in tumors. We pro-

pose that HoxA9 constitutes a new tumor suppressor gene that functions to restrict the malignant and metastatic behavior of transformed MECs in culture by regulating the expression of molecules that orchestrate MEC integrin-ECM interactions and that this modulation is dependent on the biophysical properties of the ECM.

Global expression analysis and q-RT-PCR analysis were used to identify and verify that HoxA9 expression was significantly reduced in primary breast tumors and cell lines. We investigated the inhibition by HoxA9 of the tumorigenic and metastatic behavior of two breast cancer cell lines in vitro using standard tissue culture techniques. Cells re-expressing HoxA9 were established, and the effects of HoxA9 on cell behavior were analyzed in monolayer and in 3D using growth, motility, invasion, and anchorage-dependence assays. Effects on tumor morphology were analyzed in 3D, assaying for colony size, re-establishment of adherens junctions and tissue polarity, and lumen formation. We characterized the effects of HoxA9 expression on beta-1 and beta-4 integrin expression and function using FACs analysis and adhesion assays. Polyacrylamide gels of various stiffnesses were used to investigate the influence of matrix mechanics on HoxA9 modulation of breast tumor phenotype.

Thus far, we have shown that HoxA9 is lost in invasive human breast tumors and that re-expressing HoxA9 in breast cancer cells decreases migration, invasion, and anchorage-independent growth in culture. Furthermore, HoxA9 mediates the phenotypic reversion of breast tumor cells in 3D basement membrane cultures and inhibits tumorigenesis in vivo by regulating BRCA1 expression and normalizing integrin expression and extracellular matrix adhesion. Interestingly, we found that HoxA9 levels decrease significantly when MECs interact with a stiff matrix and that increased matrix rigidity compromises HoxA9-dependent phenotypic tumor reversion. Because matrix stiffening precedes and can promote malignant transformation in vivo and preventing matrix stiffening inhibits tumorigenesis, we have begun to study functional links between matrix mechanics, HoxA9, and breast cancer progression.

The identification of HoxA9 as a tumor suppressor gene along with a clear understanding of the gene's role in breast tumorigenesis and metastasis will provide a solid foundation for downstream clinical impact, including the identification of therapies targeting HoxA9 and the pathways through which it acts. Additionally, a defined relationship between HoxA9 and breast cancer metastasis may contribute to the early identification of primary breast tumors that are predisposed to metastasize, facilitating aggressive treatment and potentially improving patient prognosis.

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P31-11: EXPLORING THE ROLE OF 14-3-3σ IN ErbB2-MEDIATED MAMMARY TUMORIGENESIS

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ErbB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. Amplification and over-expression of the *erbB2* gene is observed in 20%–30% human primary breast cancers. MMTV-Cre/FloxNeoNeuNT (also referred as ErbB2 knock-in) transgenic mice, which express an activated ErbB2 under the transcriptional control of the endogenous *erbB2* promoter, were generated to examine ErbB2-mediated mammary tumorigenesis in a physiological context. Comparative genomic hybridization (CGH) and bacterial artificial chromosome (BAC) assay analysis of the mammary tumors from the ErbB2 knock-in mice identified a recurring 1.88 mega base pair deletion in chromosome 4, a region where the 14-3-3σ gene is located. 14-3-3σ is a putative tumor suppressor, which is transactivated by p53 in response to DNA damage. As a negative cell cycle regulator, it induces G2-M cell cycle arrest and blocks Akt-mediated cell survival and proliferation. Furthermore, epigenetic silencing of 14-3-3σ by CpG methylation was identified in over 90% primary breast carcinomas analyzed. Together, these suggest an important role for 14-3-3σ in the development of ErbB2-induced breast cancer. To understand the connection between ErbB2 and 14-3-3σ, we utilized standard biochemical and molecular approaches to study 14-3-3σ's impact on ErbB2 in vitro, and generated mammary-specific 14-3-3σ knockout mice in the ErbB2 knock-in context to examine tumorigenesis in vivo.

We have demonstrated that EGR2 (Early Growth Response 2), a zinc finger transcriptional regulator of ErbB2, associates with 14-3-3σ. The over-expression of 14-3-3σ in TM15 cell line, which is derived from a ErbB2 knock-in mammary tumor, led to a relocalization of EGR2 from the nucleus to the cytoplasm and a down-regulation of ErbB2 levels, which suggests a mechanism that 14-3-3σ down-regulates ErbB2 at transcript level by sequestering its transcription factor EGR2 in the cytoplasm.

We have also discovered a new function of 14-3-3σ in the maintenance of cell polarity. Its expression can restore the disrupted cell junctions in TM15 cells. Its over-expression promoted the formation of polarized structures in 3-dimensional (3D) collagen cultures of MDCK cells in the context of either HGF (hepatocyte growth factor) treatment or activated ErbB2 expression. Its down-regulation by RNA interference resulted in the formation of more depolarized structures in 3D collagen cultures of MDCK cells and 3D Matrigel cultures of MCF10A cells.

Meanwhile, mammary-specific 14-3-3σ knockout mice with uniform FVB/N background have been made and cohorts of the mammary-specific 14-3-3σ knockouts in the context of activated ErbB2 expression are also under generation. Histological and wholemount analysis of mammary gland development and close monitoring of mammary tumor onset by biweekly palpation are currently being carried out.

In summary, we discovered the role of 14-3-3σ in ErbB2 down-regulation and cell polarity maintenance, and we generated mammary-specific 14-3-3σ knockout mice, which are being characterized. This research will help to further understand the mechanism of ErbB2-mediated tumorigenesis. The result may disclose some key factors that mediate the tumor onset and/or metastasis, which can be used as pharmaceutical targets to develop medicines and contribute to the prevention and treatment of breast cancers.

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P31-12: TIGHT JUNCTION PROTEINS OCCLUDIN AND ZO-1 ARE SUBSTRATES OF THE RECEPTOR PROTEIN TYROSINE PHOSPHATASE DEP-1

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DEP-1 is a receptor protein tyrosine phosphatase (PTP) that has been mapped to the mouse colorectal tumor susceptibility locus *Sccl*. The loss of heterozygosity of the DEP-1 gene has been found in several human cancers such as breast and lung (Ruijvenkamp et al. 2002). In vivo, DEP-1 is localized to the plasma membrane and concentrates at points of cell-cell contact. Tyrosine phosphorylation of junctional proteins is known to destabilize cell-cell junctions, and destabilization of cell junctions is associated with tumor cell invasion and metastasis. The localization pattern of DEP-1 suggests that it can regulate the phosphorylation level of proteins at cell-cell adhesions and therefore regulate junction integrity. In this study, we have found that the receptor PTP DEP-1 can bind to two members of the tight junction, occludin and ZO-1. Occludin and ZO-1 bind in a tyrosine phosphorylation-dependent manner via the catalytic domain of DEP-1 and not by other protein-protein interaction motifs. Wild-type DEP-1 is able to dephosphorylate occludin and ZO-1, further supporting occludin and ZO-1's role as substrates. In addition, these interactions with DEP-1 seem to be specific since other PTPs tested were not able to bind to occludin and ZO-1 nor did they dephosphorylate them. Future work will look at the function of these interactions in forming and maintaining cell-cell contacts. By controlling phosphorylation levels of tight junction proteins, DEP-1 may enhance the stability of cell-cell junctions and thereby inhibit loss of cell-cell adhesion associated with epithelial-to-mesenchymal transition. Learning more about the basic biology of DEP-1 should help to clarify its role as a tumor suppressor gene and in cancer biology.

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P31-13: SUPPRESSION OF THE NEGATIVE REGULATOR LRIG1 CONTRIBUTES TO ErbB2 OVEREXPRESSION IN BREAST CANCER

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The ErbB family of receptor tyrosine kinases (RTKs), which includes EGFR, ErbB2/Her2, ErbB3, and ErbB4, are key mediators of normal processes, such as the development and maintenance of tissues, and are also critically involved in the growth and progression of human tumors. Overexpression of these receptors, particularly ErbB2 which is overexpressed in 25%–30% of breast tumors, leads to constitutive activation, resulting in enhanced signaling through pathways that promote tumor progression. Recently, the leucine-rich repeat protein LRIG1 has been shown by our lab to interact with and downregulate all four ErbB family members independent of growth factor stimulation by enhancing receptor ubiquitination. Given the enhanced expression of these receptors in breast cancers, we propose that LRIG1 is a negative regulator of ErbB function in mammary carcinomas and is downregulated or lost in a subset of these tumors. In the present study, we demonstrate that LRIG1 protein levels are significantly suppressed in the majority of ErbB2-positive human breast tumors, raising the possibility that LRIG1 loss could contribute to the initiation or growth of ErbB2-positive breast tumors. Additionally, RNAi-mediated knockdown of endogenous LRIG1 in breast tumor cell lines further elevates ErbB2 and augments cellular proliferation. In contrast, ectopic expression of LRIG1 reverses these trends. Taken together, these data indicate that LRIG1 may help keep cellular ErbB2 levels balanced and that the loss of this protein may be involved in breast tumor progression.

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P31-14: CHFR IS A NOVEL TUMOR SUPPRESSOR, REGULATOR OF GENOMIC INSTABILITY, AND CHEMOTHERAPEUTIC BIOMARKER IN BREAST CANCER

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Background: Breast cancer can develop when genes that control the cell cycle and genomic stability are aberrantly expressed or become nonfunctional. *CHFR* encodes an E3 ubiquitin ligase protein that reportedly delays progression through prophase in response to microtubule-targeting taxane drugs (i.e., nocodazole, paclitaxel). Loss of *CHFR* mRNA expression has been reported in many cancers compared to normal tissues, but few have tried to explain the importance of this in tumorigenesis. The normal cellular function of *CHFR* also is poorly understood. Our goals were to: (1) evaluate *CHFR*'s role in breast tumorigenesis, (2) investigate its potential as a biomarker for chemotherapeutic response or prognosis, and (3) identify new *CHFR* protein interactions relevant to oncogenesis.

Methods: Using immunohistochemistry, we examined correlations between *CHFR* expression and clinico-pathological data in 160 primary invasive breast cancers and tested expression in 27 mammary epithelial cell lines by western blotting. We knocked down *CHFR* expression in two nontumorigenic immortalized human mammary epithelial cell (IHMEC) lines using RNAi and overexpressed *CHFR* in the Hs578T breast cancer line, then tested these models for phenotypic changes relevant to cancer. We studied chromosome segregation using immunofluorescence. Novel *CHFR* protein interactions were identified and characterized by immunoassay techniques.

Results: Nearly 40% of cultured and primary breast cancers had low or no *CHFR*, which was associated with large tumor size and weakly correlated with ER(-) tumors. *CHFR* knockdown in IHMEC lines resulted in increased taxane sensitivity and the acquisition of tumorigenic phenotypes including faster growth rates, higher mitotic indices, enhanced invasiveness and motility, epithelial-to-mesenchymal transitions, aneuploidy, and colony formation in soft agar. Conversely, overexpressing *CHFR* in breast cancer cells caused slower growth and decreased invasiveness and motility. Transient *CHFR* loss quickly led to aneuploidy and the mitotic defects of misaligned metaphase chromosomes, anaphase bridges, multi-polar condensed spindles, multinucleated cells, and mislocalization of the mitotic checkpoint proteins MAD2 and BUBR1. We identified *CHFR* interactions with MAD2 and Aurora A where *CHFR* loss led to elevated Aurora A oncoprotein levels but no change in MAD2 expression. α -tubulin was identified as a novel target for *CHFR*-mediated ubiquitination and degradation after treatment with nocodazole. Decreased *CHFR* increased the amount of acetylated α -tubulin, a mitotic spindle protein that has been implicated in cellular response to taxane treatment.

Conclusions: *CHFR* is a potent tumor suppressor in breast cancer cell culture models, and its expression may predict tumor sensitivity to taxane treatment. Our data suggest that *CHFR* has a previously unrecognized role as a regulator of genomic stability via its functional impact on BUBR1, MAD2, Aurora A, and α -tubulin. Our discovery that *CHFR* contributes to the modification and turnover of α -tubulin suggests a novel mechanism for how *CHFR* responds to taxanes and/or regulates mitotic spindle formation. *CHFR* may be one of the few genes that can control the cell cycle, cellular response to drug treatment, and genomic stability—processes that go awry in breast cancer.

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P31-15: PROAPOPTOTIC BimEL IS DEGRADED BY β TRCP1/2 UBIQUITIN LIGASE COMPLEX

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BimEL (Bcl-2 interacting mediator of cell death, extra long isoform) is a proapoptotic member of the Bcl-2 protein family that links stress-induced signals to the apoptotic machinery. Induction of survival signals such as the addition of growth factors promotes the ubiquitination and proteasomal degradation of BimEL in a phosphorylation-dependent manner. Despite BimEL's evident importance in determining cell fate, the cellular machinery responsible for its degradation has not yet been identified. In this work, we demonstrate that SCF ^{β TRCP1/2} is the ubiquitin ligase complex governing BimEL protein levels. Binding of β TRCP1/2 to BimEL is dependent on phosphorylation of BimEL on three serine residues found in a consensus degron sequence similar to other β TRCP1/2 substrates. In addition, we show that phosphorylation of these residues is carried out by Rsk1. Our data link the ubiquitin ligase β TRCP1/2 and the kinase Rsk1 to the intrinsic apoptotic pathway via regulation of the protein levels of BimEL. It was recently shown that paclitaxel-induced cell death of epithelial cells is mediated via BimEL. Interestingly, when H-ras/MAPK pathway is activated in tumors, BimEL expression is suppressed by phosphorylation and subsequent degradation, allowing

tumor cells to escape chemotherapy-induced cell death. Therefore, targeting the Rsk1- β TRCP1/2-BimEL axis has the potential to help sensitize tumor cells to chemotherapy. We are now testing this hypothesis in different breast cancer cell line models.

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P31-16: THE TUMOR SUPPRESSOR ACTIVITY OF THE LYSYL OXIDASE PROPEPTIDE REVERSES THE INVASIVE PHENOTYPE OF Her-2 DRIVEN BREAST CANCER AND IS IMPAIRED IN POLYMORPHIC VARIANT

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Expression of the *lysyl oxidase* gene was found to inhibit the transforming activity of the *ras* oncogene in NIH 3T3 fibroblasts and was hence named the "ras reversion" gene (*rrg*). Lysyl oxidase (LOX) is synthesized and secreted as a 50 kDa inactive pro-enzyme (Pro-LOX), that is processed by proteolytic cleavage to a functional 32 kDa enzyme and an 18 kDa propeptide (LOX-PP). Recently, the *ras reversion* activity of the *lysyl oxidase* gene in NIH 3T3 cells was mapped to its propeptide region (LOX-PP). Here, we tested the hypothesis that LOX-PP inhibits transformation of breast cancer cells driven by Her-2/neu, an upstream activator of Ras. We show that LOX-PP expression in Her-2/neu-driven breast cancer cells in culture suppressed Akt, Erk, and NF- κ B activation. Epithelial to mesenchymal transition (EMT) is a process implicated in the progression of primary tumors toward metastasis. Her-2/neu-induced EMT was reverted by LOX-PP, as judged by reduced levels of Snail and vimentin, upregulation of E-cadherin, γ -catenin and estrogen receptor (ER) α , and decreased ability to migrate or to form branching colonies in Matrigel. Furthermore, LOX-PP inhibited Her-2/neu-induced tumor formation in a nude mouse xenograft model. Thus, LOX-PP inhibits signaling cascades induced by Her-2/neu that promote a more invasive phenotype and may provide a novel avenue for treatment of Her-2/neu-driven breast carcinomas.

Two single nucleotide polymorphisms (SNPs) in the propeptide region of Pro-LOX were reported in gastric cancers. Introduction of the equivalent mutations in murine LOX-PP resulted in less effective suppression of Her-2/neu signaling, markedly impaired ability to reverse invasive phenotype in vitro and to prevent tumor formation in vivo. These findings suggest that genetic variation is a mechanism of loss of *rrg* activity of the *lysyl oxidase* gene, which may lead to increased susceptibility to invasive breast cancer.

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P31-17: THE PROLYL ISOMERASE Pin1 MODULATES RETINOBLASTOMA PROTEIN IN RESPONSE TO IRONIC IRRADIATION

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The tumor suppressor retinoblastoma protein (Rb) plays a critical role in human tumorigenesis. Inactivation of Rb has been well documented in breast cancer. While Rb plays a pivotal role in cell cycle G1-S transition, emerging evidence indicates that Rb is also critical for S-phase checkpoint control in cellular response to DNA damage. The prolyl isomerase Pin1 has been implicated in regulation of cell cycle progression and in cellular response to DNA damage. Mice lacking Pin1 show defects in mammary development. In addition, Pin1 overexpression has been observed in human breast cancers, suggesting that Pin1 may play a role in mammary tumorigenesis. The purpose of this study is to test our hypothesis that Pin1 binds to and inhibits Rb during mammary tumorigenesis. Here, we show that CDK-mediated phosphorylation of Rb facilitates its interaction with Pin1. Pin1 in turn inhibits protein phosphatase 2A (PP2A)-mediated Rb dephosphorylation, attenuates Rb dephosphorylation upon S-phase DNA damage, and compromises the ionic-irradiation induced S-phase checkpoint control. Ablation of Pin1 leads to a significant increase in hypophosphorylated Rb, accelerated Rb dephosphorylation, and increased cell growth arrest upon S-phase DNA damage. Furthermore, Pin1 inhibits Rb dephosphorylation at mitotic exit. Moreover, Pin1 is overexpressed concomitant with hyperphosphorylated Rb in human breast cancers. Together, these data reveal a novel regulatory pathway for Rb in which Pin1 is critical for modulation of Rb function in S-phase checkpoint control upon DNA damage.

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P31-18: INHIBITION OF MAD1 BY AKT PROMOTES HER-2 ONCOGENIC SIGNALING

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The amplification and/or overexpression of oncogene *HER2/neu* is found in approximately 25% of human breast cancers and often associated with poor prognosis including short survival and relapse time. Previous studies have revealed that *HER2/neu* can enhance MYC stability and activity through RAS/RAF/ERK pathway. In addition, PI3K/AKT pathway acts in the downstream of *HER2/neu* signaling and regulates many tumorigenesis pathways including up-regulation of many MYC target genes. However, the detail mechanism of AKT-MYC pathway is obscure. MAD1 is a bHLH-ZIP transcription factor and form heterodimer complex with MAX that plays a role in opposing MYC-MAX heterodimer with respect to the regulation of gene expression. According to the literature, several AKT downstream molecules contain the RXXRX(S/T) motif that can be recognized and phosphorylated by AKT. Analyzing the molecules participating in MYC signaling network, we found that MAD1, an antagonist of MYC, contains this motif and could be a downstream substrate of *HER2/neu*/PI3K/AKT signaling pathway. Here, we found that overexpression of constitutive active AKT inhibited MAD1 function and resulted in up-regulation of MAD1 target genes. In addition, AKT can physically interact with MAD1 and subsequently phosphorylate MAD1 at Ser145. To mimic different status of MAD1 phosphorylation, Ser145 was changed to either aspartic acid (D) or alanine (A) to mimic phosphorylation or non-phosphorylation, respectively. Our results indicated that MAD1(S145A) mutant enhanced its binding affinity with MAX, suggesting that phosphorylation of MAD1 at Ser145 will decrease MAD1-MAX interaction. This leads to the consequence of fading MAD1 function in MYC antagonism. Indeed, MAD1(S145D) mutant showed less ability in suppression of its target genes transcription in comparison to MAD1(S145A) mutant. This effect was also found in the transcriptional regulation of human telomerase reverse transcriptase (hTERT), a MAD1 target gene. In fact, our data demonstrated that the reduction of the interaction between MAD1 and hTERT promoter interaction after AKT phosphorylation; however, MAD1(S145A) mutant could rescue such effect. Moreover, MAD1(S145D) mutant performed less function in tumor suppression in comparison to MAD1(S145A) mutant in tumorigenesis assay. Taken together, our results demonstrated that MAD1 is phosphorylated and inhibited by AKT and the phosphorylation will reduce MAD1 function in *HER2/neu* promoted breast cancer. Therefore, MAD1(S145A) mutant, a gene could be resistant to AKT inhibitory effect, could serve as a potential role in anti-breast cancer gene therapy.

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P31-19: THE ROLE OF PinX1 IN TELOMERE REGULATION AND TUMORIGENESIS

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Background: Telomere maintenance is essential for protecting chromosome ends, and aberrations in telomere length have been implicated in cancer and aging. Excessive telomere elongation is inhibited by telomere repeat binding factor 1 (TRF1) and its associated proteins. However, the mechanism is unclear because telomere-binding proteins do not directly bind or affect telomerase, the nucleoprotein complex that elongates telomeres. We previously identified PinX1, a TRF1-interacting partner and a conserved telomerase inhibitor. PinX1 may act as the bridge between TRF1 and telomerase since it binds and inhibits telomerase through a C-terminal telomerase inhibitory domain (TID). Depletion of PinX1 in cells increases telomerase activity and tumorigenicity in nude mice while overexpression of PinX1 or its TID forces cancer cells into growth crisis and senescence. This suggests that PinX1 may be a tumor suppressor, which is supported by the location of PinX1 on chromosome 8p23, a region with loss of heterozygosity in many cancers, including breast cancer.

Objectives: We have identified several genetic alterations of conserved residues within the PinX1 TID in breast cancer samples indicating the importance of this region in oncogenesis. We are interested in determining the significance of these mutations in order to define the region of PinX1 responsible for its interactions with TRF1 and hTert, its telomere localization, and its telomerase inhibitory activity. This will allow us to elucidate a model for TRF1, PinX1, and hTert interactions in telomere dynamics and tumorigenesis.

Methods: We generated several PinX1 point mutants corresponding to genetic alterations identified in breast cancer samples, as well as a series of truncations to further define the minimal TID. GST-PinX1 fusion proteins were purified for binding assays with hTert or TRF1 and for studying telomerase inhibition in telomere repeat amplification protocol (TRAP) assays. PinX1 mutant localization was monitored by GFP-fusion protein expression. Stably overexpressing PinX1 mutant cell lines were generated to study the effects of PinX1 mutations on telomere length.

Results: The genetic alterations found in breast cancer samples did not abolish the ability of PinX1 to bind to TRF1 and to localize to telomeres or to bind to hTert and to inhibit telomerase activity. However, we have identified a 20-amino acid minimal domain directly adjacent to that region that is sufficient for binding, telomerase inhibition, and localization. Upon further mutational analysis of conserved residues, we have identified several critical residues that are important for the TRF1-PinX1 interaction and telomeric localization. Importantly, the mutations of the residues did not affect the ability of PinX1 to bind and inhibit telomerase but abolished the ability of PinX1 to induce telomere shortening when stably overexpressed in human cancer cells.

Conclusions: PinX1 recruitment to telomeres by TRF1 is critical for telomere length regulation and may provide negative feedback to telomerase to help maintain telomere homeostasis. Since upregulation of telomerase is common in many cancers including breast cancer, the identification of the minimal 20-amino acid TID that binds TRF1 and also binds and inhibits telomerase could result in the development of a potential cancer therapeutic.

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P31-20: A BONE MORPHOGENETIC PROTEIN ANTAGONIST AS A BREAST CANCER GROWTH SUPPRESSOR

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Bone morphogenetic proteins (BMPs) are extracellular signaling molecules that play important roles both in development and homeostasis of normal adult tissues. Recent data have linked multiple BMP pathways to breast cancer tumorigenesis. This project focuses on regulation of the BMP signaling pathway in breast cancer via the antagonist named sclerostin domain-containing 1 (SOSTDC1). Current knowledge concerning SOSTDC1 (also known as USAG-1, ectodin, or wise) has focused on its expression in the murine kidney, bones, and teeth and potential roles in progression of nephropathies. However, there are no published reports on the expression or action of SOSTDC1 in breast cancer. We have observed that the SOSTDC1 message is down-regulated in approximately 90% of breast cancers. We then hypothesized that SOSTDC1 is a repressor of carcinogenesis in breast tissue through extracellular antagonism of BMP signaling. We investigated this hypothesis by developing an in vitro model of SOSTDC1's expression and actions, analyzing the expression SOSTDC1 protein in breast cancer tissue samples and making recombinant SOSTDC1 protein for further assessment of its biologic properties.

Using a variety of breast cancer cultured cell lines and immunostaining with a novel antibody produced against SOSTDC1, we have observed that mature SOSTDC1 is secreted from the cell. Furthermore, in a multiple cell line model of breast cancer progression, less SOSTDC1 is secreted from more transformed cells as determined via immunoblots. We also undertook a large study of SOSTDC1 protein expression in tumors from a sample set designed to look for markers of tumor recurrence in both African American and non-African American populations. Data from these tumor microarrays have yielded information about SOSTDC1 expression levels in relation to clinical parameters such as tumor stage, recurrence, and estrogen or progesterone receptor status.

Through transient transfection of HEK293 cells, we have established a way to produce small amounts of SOSTDC1 protein. Using this recombinant protein, we observed that SOSTDC1 is able to antagonize the signaling of both BMP2 and BMP7 in breast cancer cell cultures. Additionally, increasing the SOSTDC1 in cultures of MCF7 cells through transient transfection causes a profound inhibition of culture proliferation. Taken together, these data suggest that proper levels of secreted SOSTDC1 are important to the maintenance of proper cell signaling and proliferation. Furthermore, restoration of SOSTDC1 to breast tumors may have future therapeutic potential.

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P31-21: DACH1 IS A NUCLEOLAR PROTEIN WHOSE EXPRESSION CORRELATES WITH ESTROGEN RECEPTOR α EXPRESSION IN BREAST CANCER

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The dachshund gene product DACH1 is a cell fate determination factor that plays a key role in embryogenesis and is conserved from *Drosophila* to humans. It is a member of a gene complex that includes eyes absent (eya), eyeless, sine oculis (so), and

Six1. Dysregulation of this embryologic pathway has been shown to have a role in tumorigenesis. Six1 has been shown in several studies to have oncogenic potential while we have previously shown that DACH1 acts as a tumor suppressor in breast cancer. Analysis of over 2,000 breast tissue samples, from normal to cancer, showed decreased expression of DACH1 with increasing oncogenesis. DACH1 has also been shown to work as a tumor suppressor by inhibiting the expression of the cell cycle protein cyclin D1 and c-Jun. We examined herein the relationship between DACH1 and estrogen receptor (ER) alpha and determined the subcellular localization of the DACH1 protein in both normal and cancer tissues.

The results of immunohistochemical staining of over 2,000 breast tissue samples, ranging from normal tissue to invasive carcinoma, were analyzed. Nuclear DACH1 expression was shown to be positively correlated with ER expression in breast cancer (OR 3.338) (95% CI 3.02, 16.424). Additionally, the expression of DACH1 within the cytoplasm was shown to be inversely correlated with ER expression (OR 1.675) (95% CI 1.357, 2.067). Immunofluorescent staining was performed with AQUA/

PM2000 platform on breast tissue arrays to determine the subcellular localization of DACH1 and how this changes in normal tissue versus cancer and ER-positive cancer versus ER-negative cancer. Immunofluorescent staining of breast tissue revealed that in normal breast tissue, DACH1 expression is localized to the nucleolus, whereas in cancer tissue, its expression varies from nuclear to cytoplasmic. These findings were confirmed by co-staining with nucleolar protein nucleophosmin. The binding of DACH1 to two nucleolar proteins was examined by immunoprecipitation, which reveals direct binding of DACH1 to the nucleolar proteins nucleophosmin and nucleolin. The expression of the cell fate determination factor DACH1, a known tumor suppressor in breast cancer, is correlated with the expression of ER. DACH1 is expressed exclusively in the nucleolus in normal breast tissue but is non-nucleolar in breast cancer. The cytoplasmic distribution in cancer varies with the ER status of the tumor.

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BRCA1 AND BRCA2 TUMOR SUPPRESSORS I

Poster Session P32

P32-1: BRCA1 SOMATIC METHYLATION AND EARLY ONSET BREAST CANCER

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Background and Objectives: About 75% of breast cancers that have a typical BRCA1-like histology (high-grade, high-mitotic count, solid architecture, and prominent lymphocytic infiltrates) do not harbor a somatic mutation in BRCA1. Methylation of the BRCA1 promoter is an epigenetic mechanism for functionally inactivating BRCA1 and has been reported to occur in up to 30% of sporadic breast cancers. Germline methylation has been shown to affect one of the genes underlying hereditary non-polyposis colorectal cancer, MLH1. This study set out to determine BRCA1 methylation in the peripheral blood DNA from women with early onset breast cancer without germline BRCA1 (or BRCA2) mutations and to relate this to their pathology.

Brief Description of Methodologies: We analyzed peripheral blood from patients with early onset breast cancer, both with typical BRCA1-like features (49 patients) and without typical BRCA1 like features (33 patients). DNA was analyzed for BRCA1 promoter methylation using methylation-sensitive, high-resolution melting and MethyLight. We also studied 33 control blood samples.

Results to Date: We anticipated detecting a small subset of patients with 50% methylation corresponding to methylation of a single allele. To our surprise, we found a group of patients with methylation levels between 0.1 and 10%. Seventeen of the 49 patients with BRCA1-like features had detectable BRCA1 methylation in their peripheral blood. Only 4 of the 33 patients without BRCA1-like features had detectable BRCA1 methylation in their peripheral blood. Only one of the 33 control individuals had any detectable methylation in their peripheral blood. Tumor material was tested for 20 of the patients with BRCA1-like features and 8 samples (40%) showed BRCA1 methylation. Moreover of the 5 patients with detectable BRCA1 methylation in their peripheral blood, all had BRCA1 methylated tumors. There was thus a strong implication that the methylation observed in the peripheral blood was a marker for an underlying somatic methylation of BRCA1 that then directly predisposed to breast cancer.

Conclusions: These results demonstrate that in some cases of breast cancer, promoter methylation of BRCA1 by occurs in normal tissues of the body and is associated with the development of BRCA1-like breast cancer. Individuals with detectable BRCA1 methylation in their peripheral blood may be at an increased risk for breast cancer. They may benefit from regular monitoring for signs of early disease.

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P32-2: HETEROCHROMATIN INSTABILITY IN CANCER AND POTENTIAL ROLE OF BRCA1 IN PERICENTRIC HETEROCHROMATIN

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Background and Objectives: We very recently received a concept award to study the potential compromise of heterochromatin in breast cancer and the potential link of BRCA1 to maintenance of constitutive heterochromatin. The BRCA1 tumor suppressor is implicated in multiple nuclear functions and is an ubiquitin ligase. In irradiated nuclei, BRCA1 foci form at sites of DNA repair; however, our findings have implicated the BRCA1 foci in normal, non-irradiated S-phase nuclei in replication of constitutive heterochromatin. In studying BRCA1 in relation to XIST RNA and X inactivation, we discovered that many normal BRCA1 foci (in human or mouse) directly abut or overlap markers of the interphase centromere/kinetochore complex. Thus the reported relationship of BRCA1 to Xi may reflect a broader link to heterochromatin on chromosomes generally. BRCA1 shows a striking relationship to mouse chromocenters (clustered centromeres), and this is temporally linked to their replication. Further, our findings raise the possibility that failure to maintain the Xi in many breast cancers reflects broad compromise of the nuclear heterochromatic compartment. Therefore, our goal is to investigate (1) the hypothesis that BRCA1 has a fundamental but unrecognized role in maintaining silent heterochromatic structure, particularly at centric/pericentric DNA and (2) innovative methods to examine the potential for broad compromise of heterochromatin in certain cancers, particularly breast cancer.

Methods: We are using high-resolution molecular cytological techniques, including immunofluorescence to specific proteins and in situ hybridization to DNA or RNA sequences. Numerous heterochromatin markers are examined relative to centromeric satellites. We have developed a method to assess overall integrity of the nuclear peripheral heterochromatic compartment (PHC) which utilizes hybridization to hnRNA (using Cot-1 DNA). We also have developed a method to examine expres-

sion of repeats throughout the genome. Several normal and breast cancer human cell lines are being investigated.

Results: As funding was just received, work is in progress and any results are preliminary. To determine if BRCA1- breast cancer shows defects in centromere structure, we are examining histone H3 lysK9, methK27, HP1, H4deacetylation, condensation, and nuclear organization, and silencing of satellite DNA on centromeres. Thus far, we have examined several CEN-P proteins in a BRCA1- breast cancer line, and these appeared to retain a normal relation to centromeres. Since BRCA1 is reported to ubiquitinate topoII, we recently found that topoII is also concentrated on normal mouse chromocenters during their replication, similar to BRCA1. Further results indicate chromocenters are ubiquitinated during the cell cycle, thus BRCA1 could be involved in this ubiquitination linked to replication. Thin bridges of DNA that often connect G1 daughter cells in BRCA1- cancer cells typically contain satellite DNA, which apparently fails to separate. An assay to survey the integrity of the PHC suggests that loss of Xi heterochromatin may occur in the context of broad breakdown of the nuclear heterochromatic compartment.

Conclusions: Broad breakdown of heterochromatin may be more common and important in cancer than is widely recognized, and BRCA1 may have an unanticipated role linked to maintenance of pericentric heterochromatin, with implications for both epigenomic and genomic instability.

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P32-3: BRCA1-MEDIATED MONOUBIQUITYLATION: EFFECT ON NUCLEOSOME DYNAMICS

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Mutational inactivation of the breast cancer susceptibility gene (BRCA1) accounts for 50%-90% of hereditary breast and ovarian cancers. Therefore, the protein product of BRCA1 must function to protect normal cells from becoming cancerous. BRCA1 has been implicated in multiple pathways that preserve genome stability, including cell cycle control, DNA repair, transcription, and chromatin remodeling. The multifunctional nature of BRCA1 has raised the possibility that the protein may employ a common mechanism, such as reorganizing chromatin structure, to regulate various chromosomal events. Indeed, BRCA1 is associated with histone-modifying enzymes (p300 and HDAC) and ATP-dependent chromatin remodeling machines (hSWI/SNF). Many cancer-predisposing mutations reduce BRCA1's affinity for these chromatin-modifying proteins, suggesting that chromatin remodeling may be an important aspect of BRCA1 tumor-suppression activity. BRCA1 can also function via an alternative mechanism by directly affecting nucleosome structure and dynamics through its proven protein ubiquitylation activity. It has been shown that the RING finger of BRCA1 confers ubiquitin ligase activity to the molecule, markedly enhanced when BRCA1 forms a complex with another RING protein BARD1(BRCA1 associated RING domain); the BRCA1/BARD1 complex mono-ubiquitylates purified core histones in vitro. Whether core histones can be modified in the context of chromatin is unclear. The possible role of such modification in transcription and DNA repair is also uncertain.

Thus, it is highly significant to understand the effect of BRCA1-mediated ubiquitylation of core histones on nucleosome dynamics. As a first step toward such an understanding, we have set up an in vitro system to demonstrate the ubiquitylation activity of full-length recombinant BRCA1/BARD1 complex using recombinant human H2A histone as a substrate. Ubiquitylated H2A reaction products were visualized by western blotting using both anti-H2A (Abcam) and anti-Ub (Upstate) antibodies. The results demonstrate a dual E3 ubiquitin-ligase activity of BRCA1: it modifies H2A and auto-ubiquitylates itself. The development of the in vitro system using full-length BRCA1/BARD1 complex provides an invaluable tool for further investigation of the effect of BRCA1-dependent ubiquitylation activity on nucleosome dynamics. The goal now will be to use a sensitive single-molecule assay, single-pair Fluorescence Resonance Energy Transfer (spFRET) to study (1) the spontaneous dynamics of ubiquitylated canonical nucleosomes in real time and (2) transcription elongation through such ubiquitylated nucleosome particles.

The demonstration that BRCA1 functions as an E3 ubiquitin ligase in vitro may be relevant to its role as a key regulator for maintaining genomic stability and to its participation in a diverse range of cellular processes that involve chromatinized DNA templates. Understanding the molecular mechanisms of action of BRCA1 on chromatin structure and function will be key to our overall understanding of its role in normal cell functioning. Such understanding may provide clues toward the design of new targeted anti-cancer therapeutics aimed at restoring BRCA1's normal activities.

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P32-4: EXTRA-CHROMOSOMAL CIRCULAR DNA IN BREAST CANCER: ROLE OF BRCA GENES AND EFFECT OF CHEMOTHERAPY

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Genomic instability is a hallmark of cancerous cells including breast cancer. An intriguing aspect of it is the formation of extra-chromosomal circular DNA (eccDNA). Various lines of evidence suggest that eccDNA is correlated with homologous recombination and double strand break (DSB) repair. Our working hypothesis was that elevation of eccDNA levels reflects enhanced processes (such as DNA recombination) that can augment genomic instability. The BRCA1 and BRCA2 genes play a role in homologous recombination. In addition, drugs that interfere with processes of chromosomal dynamics have chemotherapeutic potential. Therefore, we proposed to examine the feasibility of using eccDNA as a novel marker for diagnosis and efficacy of treatment of breast cancer.

Two objectives were proposed for the 1-year funding:

1. Does the DNA repair machinery that involves BRCA1 and BRCA2 participate in the formation of eccDNA?
2. Do chemotherapeutic drugs that act as inhibitors of specific genes implicated in genome stability alter the levels of eccDNA?

Our main experimental method was a 2-dimensional (2D) gel electrophoresis technique that allows separation of DNA molecules according to their size and structure.

1. The first objective was addressed by examining the level of eccDNA in *Drosophila* mutants defective in DSB repair. For each tested gene, we used available mutants and performed the appropriate genetic crosses to compare DNA from mutant flies or larvae (either homozygous, hemizygous or hetero-alleles) with their heterozygous (i.e., "normal") siblings.

Results: Mutants in several genes (including *mus101*, *rad50* and *mre11*) that are implicated in the BRCA pathway of DNA repair did not exhibit altered eccDNA levels, except for *mus209* – the *Drosophila* homolog of PCNA. Yet, the assay has many limitations including the delayed development and sick phenotype of strong mutants while weaker genetic combination showed no effect. Genetic analysis in cultured cells should replace the "whole organism" approach and may yield reliable results since eccDNA formation and maintenance occur at the cellular level.

- 2a. The second objective was addressed by exposing cultured *Drosophila* cells to drugs. **Results:** We found that the histone deacetylase inhibitor Trichostatin A (TSA) alters eccDNA levels in cultured cells. This was accompanied by either differentiation or death of the cells. TSA and its derivatives have recently shown encouraging results as anti-cancer drugs. Our finding implicated chromosome structure in the machinery of eccDNA formation and indicates that eccDNA levels may serve as a marker for drug response.

- 2b. To expand the cell culture approach to human cells, we established the optimal conditions for eccDNA analysis of such cells.

Results: We detected eccDNA homologous to alpha satellite and 5S rDNA in HeLa and in HEK 293 cells. This sets the grounds for examining eccDNA machinery in cultured human cells as we already started to do with *Drosophila* cells.

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P32-5: ROLE OF THE BRCA2 CARBOXYL-TERMINUS IN Rad51-MEDIATED HOMOLOGOUS RECOMBINATION

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The tumor suppressor BRCA2 functions by enhancing the efficiency of the homology-directed repair of DNA double-strand breaks. Recent biochemical evidence has revealed that BRCA2 acts by nucleating the Rad51 recombinase onto ssDNA and enabling Rad51 to utilize ssDNA coated with replication protein A (RPA) as recombination substrate. The interaction with Rad51 is mediated by two distinct domains in BRCA2 – the BRC repeats and carboxy terminal recombinase binding (CTRB) domain. Deletion of the CTRB domain engenders recombinational repair deficiency, and cancer mutations are found within this domain. To decipher the function of the CTRB domain in homologous recombination, we have constructed and characterized functional polypeptides of BRCA2 that harbor this domain. We will provide evidence that the CTRB domain facilitates the delivery of Rad51 to RPA-coated ssDNA.

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P32-6: BASE EXCISION DNA REPAIR DEFECTS IN BASAL-LIKE AND BRCA1-MUTATED BREAST CANCER CELL LINES

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Breast cancers of the basal-like subtype, which share a similar gene expression profile to breast cancers with BRCA1 mutations, are characterized by a "triple-negative" phenotype, i.e., negative for expression of the estrogen, progesterone, and HER2 receptors. Both basal-like and BRCA1-mutated breast cancers are often associated with aggressive disease, including poor clinical outcome, recurrence, and metastasis. The underlying mechanisms that contribute to the initiation and progression of these cancers is currently unknown but remains important for developing novel options for prevention and/or treatment. Excessive DNA damage often leads to mutations in important cancer regulatory genes. Oxidative DNA damage (ODD), the most common form of DNA damage, is induced by reactive oxygen species (ROS) and under normal conditions, is corrected by the base-excision repair (BER) pathway. In this study, we hypothesized that breast cancers of the basal-like subtype or with BRCA1 mutations exhibit an impaired defense mechanism against oxidative stress. We evaluated a panel of human breast cell lines for cellular response to hydrogen peroxide (H₂O₂) by MTT assay. We found that cell lines of the basal-like subtype were more susceptible to H₂O₂ than luminal breast cancer cell lines and normal breast cell lines. Cell lines of the basal-like subtype showed similar susceptibility to cell lines with BRCA1 mutations. We next analyzed the ability of the cancer cell lines to repair ODD by BER using a host-cell reactivation assay. We introduced ODD into a GFP reporter gene *ex vivo*, which was introduced into each cell line by adenoviral-mediated gene transfer, and found that cancer cell lines of the basal-like subtype were less efficient in activating GFP expression than luminal breast cancer cell lines but not compared to a breast cancer cell line with mutated BRCA1. Finally, MCF7 human breast cancer cells stably transfected with two different shRNAs to hOGG1, an important enzyme in the repair of ODD by BER, showed 79% and 51% knock-down of mRNA expression, were 3-fold more susceptible to H₂O₂ induced cytotoxicity, and were 81% and 54% less likely to undergo host-cell reactivation of the oxidative DNA damaged GFP reporter compared to cells transfected with a control shRNA. In addition, we developed a novel assay that provides steady-state measurements of BER activity not only *in vitro* but also *in vivo*. The assay uses a biotin-tagged oligonucleotide substrate with a single 8oxodG lesion on the untagged DNA strand. In the first step, the substrate is transfected into target cells, allowed sufficient time for repair of the 8oxodG lesion, and then recovered using pull-down with streptavidin-coated beads. In the second step of the assay, the strand containing the lesion is isolated from the streptavidin-bound strand by denaturation and then subjected to an 8oxodG competitive ELISA kit to measure the disappearance of 8oxodG lesions over time. Overall, we found that breast cancers of the basal-like subtype and with BRCA1 mutations showed a similar phenotype to cells deficient in an important BER enzyme. Our data suggest that these cancers harbor a defective response to oxidative stress and compromised BER, which may contribute to genomic instability and their aggressive phenotype. These results have implications for breast tumorigenesis, prevention, and treatment.

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P32-7: ABROGATION OF BRCC36 IMPAIRS IR-INDUCED BRCA1 ACTIVATION AND SENSITIZES BREAST CANCER CELLS TO IR-INDUCED APOPTOSIS

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Breast cancer is a genetically heterogeneous disease, and multiple genes remain to be identified among BRCA1 and BRCA2 mutation-negative breast cancer-prone families. We hypothesized that other proteins, which have equivalent or complementary functions as BRCA1, may also be involved in the development of breast cancer. We have previously reported the identification and characterization of a novel BRCA1/2 interacting protein complex BRCC (BRCA1/2 Containing Complex). BRCC36, one of the proteins in BRCC, directly interacts with BRCA1, regulates the ubiquitin E3 ligase activity of BRCC, and is aberrantly expressed in the majority of clinical breast tumors. To further elucidate the functional consequence of abnormal BRCC36 expression in breast cancer, we performed *in vivo* silencing studies using small interfering RNAs (siRNA) targeting BRCC36 in the breast cancer cell lines that constitutively express high levels of BRCC36. Since the BRCA1 protein is activated via the ATM/CHK2 signaling pathway following the exposure of cells to DNA damaging agents such as ionizing radiation (IR), we sought to determine the role of BRCC36 in this response. Knock-down of BRCC36 alone did not affect MCF7 cell growth but when combined with ionizing radiation (IR) exposure, led to an increase in apoptotic cells (45.9% ± 4.3% vs. 34.9% ± 1.9%) (*p* < 0.05) and decreased cell viability (50.9% ± 5.8% versus 58.4% ± 5.7%) when compared to the siRNA control group. Similar results were

found in T47D and ZR-75-1 breast tumor cells. Importantly, immunoblot analysis showed that BRCC36 depletion disrupted IR-induced phosphorylation of BRCA1 but had no effect on activation of ATM, p21, and p53 or BRCA1 and BARD1 interaction following IR exposure. We also evaluated the role of BRCC36 in the formation of BRCA1 nuclear foci in response to DNA damage by immunofluorescent staining. Quantification of BRCA1 nuclear foci following IR exposure showed that siRNA-BRCC36 transfection resulted in ~63% and 52% decrease compared with siRNA-control MCF-7 cells at 2h and 4h post-IR, respectively ($p<0.05$). Similar results were found in the experiments using T-47D and ZR-75-1 cells. Importantly, gamma-H2AX nuclear foci response to IR was unaffected in the cells transfected with BRCC36 siRNA. Therefore, BRCC36 abrogation prevented the formation of BRCA1 nuclear foci in response to DNA damage. Furthermore, depletion of BRCC36 by siRNA targeting significantly decreased the level of MRE11 compared to cells transfected with siRNA control. These findings suggested that the abrogation of BRCC36 potentially interferes with the activation of BRCA1 in response to DNA damage possibly by mediating the integrity of the MRN complex. In summary, down-regulation of BRCC36 expression impairs the DNA repair pathway activated in response to IR via abolishing BRCA1 activation and appears to sensitize breast cancer cells to IR-induced apoptosis.

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P32-8: NFB1, 53BP1, AND BRCA1 HAVE BOTH REDUNDANT AND UNIQUE ROLES IN THE ATM PATHWAY

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Ionizing radiation activates the ATM (ataxia-telangiectasia mutated) kinase, allowing it to activate Chk2 (checkpoint kinase 2). Chk2 then signals to proteins that initiate cell cycle arrest or apoptosis. The mechanism by which ATM activates Chk2 remains unclear. In *S. cerevisiae*, the “mediator” protein Rad9 is crucial for the Mec1 (ATM/ATR homolog) to activate Rad53 (Chk2 homolog). NFB1 (nuclear factor with BRCT domains 1), 53BP1 (p53 binding protein 1), and BRCA1 (breast cancer tumor suppressor 1) are candidate Rad9 orthologs. We hypothesized that NFB1, 53BP1, and BRCA1 have redundant functions in the ATM pathway. In primary human foreskin fibroblasts and U2OS cells, mediating ATM autophosphorylation is a role unique to 53BP1. All three mediators played a collective role in promoting IR-induced Chk2 phosphorylation. NFB1 and 53BP1 were found to be redundant in recruiting phospho-ATM, phospho-Chk2, and NBS1 to ionizing radiation-induced foci (IRIF). Furthermore, we elucidated the interplay between NFB1, 53BP1, and BRCA1 in recruitment of each other to IRIF. We found that NFB1 is upstream of 53BP1 and BRCA1 IRIF. NFB1 and 53BP1 redundantly recruit BRCA1 to IRIF. Moreover, depleting NFB1, 53BP1, and BRCA1 simultaneously caused increased apoptosis compared to the single depletions.

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P32-9: MOLECULAR BASIS FOR BRCA2-MEDIATED DNA REPAIR AND BREAST TUMOR SUPPRESSION

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Mutations in the BRCA2 gene are responsible for a significant portion of familial breast cancer cases. An involvement of BRCA2 in the repair of damaged chromosomes is evident from the observations that (1) cells deficient in BRCA2 are highly sensitive to genotoxic agents and (2) BRCA2 interacts with the RAD51 recombinase, a member of the RAD52 group of proteins that mediate the error-free repair of chromosomal breaks through homologous recombination. Deciphering the role of the BRCA2-RAD51 complex in homologous recombination reactions will enhance our understanding of its biological function and elucidate the link between impaired homologous recombination and breast tumor formation.

The main goal of this fellowship project is to define the role of DNA binding in this BRCA2 function. The BRCA2 DNA-binding domain (DBD) represents a highly conserved region within BRCA2-like molecules and harbors a significant portion of tumor-derived missense mutations, underscoring the importance of addressing the functional significance of this BRCA2 domain. Moreover, I will introduce cancer-associated mutations found in the DNA-binding region of BRCA2 in an attempt to rationalize how mutations in BRCA2 lead to genome instability and breast cancer. The knowledge gained from this study will make a significant contribution toward defining the mechanistic role of BRCA2 in breast cancer suppression and will be beneficial for breast cancer prevention, diagnosis, and treatment.

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P32-10: DISRUPTION OF BRCA2-Rad51 COMPLEX IN BREAST CANCER CELLS

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Rationale: BRCA2 is central to homologous recombinational repair (HRR) through BRC-mediated Rad51 interactions required for the assembly of DNA damage-induced RAD51 foci. Indeed, it has been shown that defective cell lines in HRR are high sensitivity to killing by cross-linking agents such as cisplatin.

Objective: Find natural compounds that will inhibit BRCA2-Rad51 interaction in order to inhibit the homologous recombinational process and consequently sensitize breast tumor cells to therapeutic agents.

Results: *Selection of BRCA2-Rad51 Candidate Inhibitors:* We have cloned the BRCA2 domain that interacts with Rad51 and full-length Rad51 and utilized these cDNA sequences to screen the panel of 15,000 natural compounds from the Chinese National Center for Drug Screening system using a yeast two hybrid system. We find 18 compounds displaying selective inhibition of BRCA2-Rad51 interaction had IC₅₀ values less than 10µg/ml.

Biological Evaluation of Candidate Inhibitors: From the compound tested, only one compound (compound 19) was soluble in DMSO at a concentration of DMSO, which was not toxic to the cells (0.05%DMSO in culture media). The biological activity of compound 19 was tested in a sporadic human breast cancer cell line panel (expressing wild-type BRCA2) and CAPAN-1, a BRCA-2-deficient cell line using the NCI-SRB assay. All tests were performed using sublethal doses of compound 19 in combination with cisplatin 0-40 µM of cisplatin.

The results obtained are summarized in the Figure. The values represent the mean value of 3 independent experiments for each cell line shown in plus minus the standard deviation. ND indicates that the cisplatin concentration range was not high enough to reach the IC₁₀ value. Statistically significant difference between the mean values was assessed using the paired t-test for means.

		CAPAN-1	MCF-7	T47D	Z75R	CAMA
Cisplatin	IC ₁₀	0.87±0.1	1.78±0.07	2336±2.06	3.63±0.82	5.23±1.17
	IC ₅₀	17.02±0.39	10.17±0.75	ND	ND	ND
Alone	IC ₁₀	0.048±0.01	0.30±0	0.54±0.13	0.16±0.049	0.28±0.84
	IC ₅₀	0.78±0.41	1.73±0.11	1947±3.43	3.2±0.2	4.43±0.06
Cisplatin + 5µM Comp19	IC ₁₀	1.25±1.19	0.00±0.3	ND	ND	ND
	IC ₅₀	0.083±0.07	0.3±0.056	1.06±0.27	0.16±0.02	0.28±0.02

Conclusion: Compound 19 when used at 5µM did not sensitize breast cancer cells to cisplatin. Nevertheless due to solubility problems, we were not able to achieve higher concentrations of the compound to effectively inhibit BRCA1-Rad51 interaction.

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P32-11: ALLELIC IMBALANCE IN BRCA1 AND BRCA2 GENE EXPRESSION IS ASSOCIATED WITH INCREASED BREAST CANCER RISK

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The contribution of *BRCA1* and *BRCA2* to familial and nonfamilial forms of breast cancer has been difficult to accurately estimate because the myriad of a potential genetic and epigenetic mechanisms that can ultimately influence their expression and cellular activities. Therefore, we investigated if allelic loss of *BRCA1* or *BRCA2* expression was associated with an increased risk of developing breast cancer. By designing allele-specific real-time PCR to target common polymorphisms, *BRCA1*-c.4308T/C or *BRCA2*-c.3396A/G, we were able to evaluate quantitatively the allelic imbalance (AI) in *BRCA1* and *BRCA2* gene expression. We first demonstrated, using mRNA from *BRCA1* and *BRCA2* frameshift mutation carriers, that the transcript ratios between the wild-type alleles to the corresponding mutant alleles (*BRCA1*-c.3671ins4 or *BRCA2*-c.796delT) were significantly increased. The observed AI is the result of non-sense-mediated mRNA decay and leads to subsequent loss of protein expression. We next measured AI for *BRCA1* and *BRCA2* in heterozygous lymphocytes from three groups, familial breast cancer patients, nonfamilial breast cancer patients, and age-matched cancer-free females. The AI ratios of *BRCA1* but not *BRCA2* in the lymphocytes from familial breast cancer patients were found to be significantly increased as compared to cancer-free women [*BRCA1*: 0.424 ± 0.157 (n=32) versus 0.211 ± 0.169 (n=40), $p=0.00001$; *BRCA2*: 0.206 ± 0.180 (n=37) versus 0.172 ± 0.123

($n=31$, $p=0.38$). Similarly, the AI ratios were greater for *BRCA1* and *BRCA2* in the lymphocytes of nonfamilial breast cancer cases versus controls [*BRCA1*: 0.353 ± 0.209 ($n=32$), $p=0.002$; *BRCA2*: 0.267 ± 0.171 ($n=26$), $p=0.03$]. Interestingly, the distribution of underexpressed alleles of *BRCA1* and *BRCA2* were found to be significantly different between cancer-free control and familial breast carriers ($p=0.02$ and $p=0.02$, respectively) but not between cancer-free control and nonfamilial breast carriers ($p=0.18$ and $p=0.20$, respectively). In conclusion, we have developed a quantitative approach to evaluate expression of *BRCA1* and *BRCA2* from individual alleles, and we have found that AI in *BRCA1* and to a lesser extent, *BRCA2*, is associated with increased breast cancer risk.

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P32-12: BRCA1 AND BRCA2 POST-TRANSCRIPTIONAL REGULATION AND CANCER RISK

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Most cases of breast cancer are sporadic, but 5-10% of breast cancers are caused by inherited mutations in breast cancer tumor suppressor genes, principally *BRCA1* or *BRCA2*. Traditionally, mutations in these genes are thought to be up to 87% penetrant for breast cancer risk. Patients with strong family histories of breast cancer are therefore considered to be at high risk for carrying a *BRCA1* or *BRCA2* mutation and may be advised to receive genetic testing. It is thus critical to know that the test results are as meaningful as possible, both to individual families and to health care providers working to understand the impact of these mutations in clinical populations.

Recent studies have shown that only 44% of patients predicted to carry mutations on *BRCA1* or *BRCA2* test positive for mutations upon screening, and only 63% of familial breast cancers genetically linked to *BRCA1* on chromosome 17 have detectable mutations in the *BRCA1* gene. These observations suggest there may be mutations in *BRCA1* or *BRCA2* that cannot be detected by current screening methods. Indeed, several studies have described large chromosome rearrangements that disrupt the *BRCA1* or *BRCA2* genes that were previously undetectable. Other types of mutations, including promoter mutations, splicing mutations, and mutations that result in mRNA instability because of defective maturation processes, have been identified in other disease-associated genes but have never been systematically sought in *BRCA1* or *BRCA2* and would likely not be recognized by conventional sequencing methods. To determine whether such mutations contribute to breast cancer risk in a clinical population, we analyzed lymphoblastoid cell lines from breast cancer patients seen at the University of Chicago Cancer Risk Clinic to determine whether heterozygous exonic single-nucleotide polymorphism (SNP) alleles in *BRCA1/2* were equally represented in the mRNA pool. This serves as a functional assay for an allele-specific mRNA-accumulation phenotype that does not require prior molecular identification of any putative message-stabilizing mutation. Of 36 cell lines previously testing negative for *BRCA1/2* deleterious mutations, none showed allele-specific defects in mRNA accumulation. While the preliminary sample size is small, we speculate that potential uncharacterized mutations associated with mRNA accumulation defects do not play a significant role in *BRCA1/2*-associated cancer risk in a risk clinic setting.

While germline mutations in *BRCA1/2* are associated with cancer risk per se, epigenetic regulation of *BRCA1/2* may influence the tumor subtype that develops during tumorigenesis. One potential mechanism of epigenetic *BRCA1/2* gene regulation is the differential regulation of alternate splice products during tumorigenesis. These variants may represent an important source of functional diversity. One of the most common *BRCA1* splice variants is an in-frame omission of the very large exon 11, resulting in a *BRCA1* protein variant. While characterizing the splice variants that could potentially vary between normal and tumor cells, we found preliminary evidence for an in-frame *BRCA2* splice variant that also lacks the very large exon 11. This variant was detected in both lymphoblastoid and breast cancer cell lines and could help refine understanding of the multiple functions of the *BRCA2* gene in tumor suppression.

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P32-13: ROLE OF IMPAIRED TGF- β SIGNALING IN DEVELOPMENT OF BRCA1-DEFICIENT BREAST CANCER: MOUSE MODEL STUDIES

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Mouse models can be employed to uncover potential mechanisms that contribute to the development of human breast cancer, to guide mechanistic studies in humans for breast cancer risk. In a mouse model of *Brc1* mutation-related breast cancer, loss of

full-length *BRCA1* expression results in down-regulation of all members of the growth inhibitory transforming growth factor beta (TGF- β) family (Jones et al., *Oncogene*, 2007, Jul 23; [Epub ahead of print]). The hypothesis that is being tested here is that loss of *BRCA1* function in mammary epithelial cells results in decreased expression of TGF- β ligands 1, 2, 3, and TGF- β receptors 1 and 2, leading to decreased activity of the TGF- β growth inhibitory pathway resulting in an exaggerated proliferative response to estrogen stimulation that precipitates cancer development. The study was funded in September 2007 and progress to date on Specific Aim 1 is reported here. First, we will establish if decreased expression of TGF- β family members is directly or indirectly related to loss of *BRCA1* in mammary epithelial cells in vivo. All the required samples from 4-month old and 10-month old *Brc1^{lff}/MMTV-Cre* mice are collected and breeding pairs set-up to generate the remaining mice required. For wild-type mice all the required samples from 6-month old cohort have been collected as well as 10 samples from 6- and 10-week old and 4-month old cohorts. All remaining number of 6- and 10-week old wild-type mice are generated and tissues are pre-planned to be collected by February 2008 for these cohorts. For the cohort of 2-week old wild-type mice, 3 breeding pairs have been set-up and tissues will be collected when ready. Second, we will test if decreased expression correlates with decreased activity of the pathway and establish how this affects the response to estrogen. *Brc1^{lff}/MMTV-Cr/6(SJL)-Tg(SBE/Tk-luc)7Twe/J* mice were acquired and the first litter has been produced and genotyping results are pending. Three, we will determine if mammary-targeted transgenic-activated TGF- β 1 expression will restore normal pubertal development and decrease the prevalence of preneoplasia and cancer. (MMTVTGFB1) 46Hlm/J, mice were acquired from the Jackson lab and 15 breeding pairs were set-up. So far, 10 breeding pairs have produced pups that will be weaned and genotyped soon. In summary, we have initiated all the experiments proposed and will present results from these experiments as available at the time of the Era of Hope meeting.

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P32-14: IDENTIFYING SUBSTRATE AND E2 INTERACTIONS OF THE BRCA1-BARD1 UBIQUITIN LIGASE

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Inherited susceptibility to breast cancer accounts for approximately 10% of all breast cancer cases and is largely attributable to germ line mutations in either *BRCA1* or *BRCA2*. *BRCA1* forms an obligate heterodimer with another protein, *BARD1*, and together they function as an ubiquitin ligase (E3), which is currently the only known biochemical activity associated with this complex. *BRCA1/BARD1* belong to the family of RING domain E3s where the RING domain interacts directly with at least one ubiquitin-conjugation enzyme (E2) to mediate transfer of ubiquitin to an E3-bound substrate. The most commonly observed cancer predisposing missense mutation of *BRCA1* (C61G) is in its RING domain and eliminates ubiquitin ligase activity. This suggests a direct link between loss of ubiquitin transfer to at least one critical substrate and breast and ovarian cancer susceptibility.

To identify the relationship between loss of ubiquitin ligase activity and breast cancer susceptibility, we set out to identify substrates ubiquitinated by *BRCA1/BARD1*. Given the paucity of reported *BRCA1* substrates, we sought a novel strategy to identify unexpected substrates and took a two-pronged approach: (1) to identify all E2s that can function with *BRCA1/BARD1* for ubiquitin transfer and (2) to ascertain whether substrates are recognized by the *BARD1* subunit, a possibility that had previously been untested.

To search for all E2s that interact and function with *BRCA1/BARD1*, a fusion protein linking the RING domain of *BRCA1* to *BARD1* was designed based on the 3D structure and screened as "bait" against 30 human E2s in directed yeast two-hybrid screens. Six novel E2s, in addition to two known interacting E2s, were found to interact with the RING of *BRCA1*. All six newly identified *BRCA1*-interacting E2s transfer ubiquitin to *BRCA1* in an auto-ubiquitination assay. Unexpectedly, the nature of the ubiquitinated product (monoubiquitin or polyubiquitin chains of specific linkage) is dictated by the E2 that interacts with *BRCA1*. Of particular import, we demonstrate that E2s known to be responsible for generation of Lys48-linked chains, which target the attached substrate for degradation by the proteasome, and of Lys63-linked chains, which are associated with DNA damage repair, are active with *BRCA1/BARD1*. These results imply that *BRCA1/BARD1* has the potential to target different substrates for different fates.

To test the possibility that *BRCA1* substrates are recognized by the *BARD1* subunit of the heterodimer, another structure-based bait was designed and screened against a human ovarian library. This screen identified a previously uncharacterized protein that is ubiquitinated in vitro in a *BRCA1* RING (E2-binding) and *BARD1* (substrate-binding)-dependent manner. We have tentatively named this protein, *BABS*, for *BARD1*-Associated *BRCA1* Substrate. However, only a subset of the *BRCA1*-interacting E2s are active toward *BABS* and either mono-ubiquitinate or polyubiquitinate,

depending on the E2 used. In directed yeast two-hybrid screens, we found that the E2s that are active with BABS also show an interaction with BABS. Our results confirm that BARD1 is utilized for substrate recognition and suggest a novel model of ubiquitination where an E2 not only transfers ubiquitin but can also be involved in substrate recognition. Additionally, as a substrate BABS may provide a link between loss-of-function mutations of BRCA1 and breast and ovarian cancer susceptibility.

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P32-15: CROSSTALK BETWEEN BRCA1 AND VITAMIN D IN GROWTH INHIBITION OF BREAST CANCER CELLS BY CO-REGULATION OF THE TUMOR SUPPRESSOR p21waf/cip

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Carriers of germline mutations in the breast and ovarian tumor suppression gene, BRCA1, have a significant increased lifetime risk for being diagnosed with breast cancer. The incomplete penetrance of BRCA1 suggests that additional environmental and/or genetic factors modify breast cancer risk and indeed, a significant delayed breast cancer onset was reported in lean mutation carriers. Thus, nutrition may profoundly alter the phenotypic expression of a given BRCA1 genotype. Some micronutrients are hormones that bind their cellular receptors and play a central role in regulation of cellular growth and differentiation. However, the preventive or causative effect of nutrition and specific micronutrients on breast cancer development among BRCA1 mutation carriers has not been explored.

We wished to determine whether VD, a fat soluble hormone and dietary factor, known to inhibit cell proliferation of normal and cancer cells collaborates with BRCA1 in growth inhibition and tumor suppression of breast cancer. We hypothesized that BRCA1 and VD work in concert to augment the growth inhibition and tumor suppression exhibited by each individual pathway.

Our hypothesis stems from the common mechanisms of action in transcriptional and chromatin structure regulation and from overlap in the list of genes regulated by BRCA1 and VD. Intriguingly, BRCA1 and VD upregulate tumor suppressor genes such as p53, p21waf/cip, and GADD45 and downregulate expression of proliferative genes such as estrogen receptor (ER) and VEGF.

Growth assays indicated that nontoxic analogues of VD promote growth inhibition of BRCA1-proficient cells (MCF7 cells up to 80%) and to much lesser extent of BRCA1-deficient cells (HCC1937, ~20%). Silencing BRCA1 expression in MCF7 cells to various degrees by shRNA technology resulted in differential cellular response to VD growth inhibition effects. The extent of BRCA1 silencing was determined by quantitative RT-PCR and by western blot analyses. While significantly silenced cells were not growth inhibited by VD, cells that were moderately silenced or transfected with nonrelevant shRNA responded to VD in a similar fashion as the parental cells.

We found that cyclin-dependent kinase inhibitor, p21^{waf/cip1}, mRNA, and protein expression are upregulated in response to VD only in BRCA1-proficient cells, and the extent of induction depends on BRCA1 expression levels. Analyzing the p21^{waf/cip1} promoter occupancy by BRCA1 and VDR using chromatin immunoprecipitation assays revealed that VDR binds to VD responsive elements (VDRE) in the p21 promoter as expected. Interestingly, BRCA1 binds to the same VDRE sites, and its association is enhanced following treatment with VD; thus, BRCA1 and VDR co-regulate p21^{waf/cip1} expression in response to VD. Co-immunoprecipitation assays indicated that BRCA1 interacts with VDR, and this interaction is enhanced following treatment with VD. In addition, we noticed that p21^{waf/cip1} promoter is significantly less acetylated on histone H4 in BRCA1-deficient cells. Taken together, our results support the hypothesis that VD cooperates with BRCA1 in tumor suppression by regulating gene expression and chromatin modifications. Studies are currently under way to identify additional BRCA1 and VDR mutual target genes that can shed light on the combined effect of nutrition and genetics.

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P32-16: BRCA1 PROMOTER METHYLATION IS ASSOCIATED WITH INCREASED MORTALITY AMONG WOMEN WITH BREAST CANCER

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Promoter-CpG island hypermethylation is a common molecular defect in cancer cells. It has been proposed as an alternative mechanism to inactivate *BRCA1* in the breast where somatic mutations of *BRCA1* are rare. To better understand breast cancer progression, we explored the association between *BRCA1* promoter methylation status and prognostic factors as well as survival among women with breast cancer. We also examined whether dietary methyl content and functional polymorphisms of genes involved in one-carbon metabolism influenced the methylation pattern. Promoter methylation of *BRCA1* was assessed from 851 archived tumor tissues collected from a population-based study with women diagnosed with invasive or in situ breast cancer in 1996–1997, and who were followed for vital status through the end of 2002. About 59% of the tumors were methylated at the promoter of *BRCA1*. The *BRCA1* promoter methylation was more frequent in cancers that were classified as invasive ($p=0.02$) and among pre-menopausal women ($p=0.05$). *BRCA1* promoter methylation was associated with increased risk breast cancer-specific mortality (age-adjusted HR 1.71; 95% CI: 1.05–2.78) and all-cause mortality (age-adjusted HR 1.49; 95% CI: 1.02–2.18). Neither dietary methyl content (folate, methionine, choline, betaine and B vitamins) nor functional polymorphisms in one-carbon metabolism were correlated with *BRCA1* methylation status. Our study is the first and largest epidemiologic investigation on the prognostic value of *BRCA1* promoter methylation from a cohort of breast cancer patients drawn from a population-based study. Our results indicate that *BRCA1* promoter methylation is an important factor to consider in predicting breast cancer survival.

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P32-17: INTERACTION OF AIB1 AND BRCA1 IN THE DEVELOPMENT OF BREAST CANCER

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AIB1 (amplified in breast cancer 1) and BRCA1 are highly relevant molecules in human breast cancer. Hereditary mutations in the *BRCA1* tumor suppressor gene confers increased lifetime risk for breast and ovarian cancers and accounts for 5%–10% of breast cancers. BRCA1 is involved in the DNA-repair process and more recently has been shown to suppress estrogen receptor transcriptional activity. Loss of these functions can lead to breast cancer. AIB1 is a steroid receptor coactivator that is amplified in 5% of primary breast tumors and overexpressed in >40% of breast tumor tissues. AIB1-delta3 is a splice variant of AIB1 that is overexpressed in breast cancer. Estrogen is the primary growth factor for the proliferation of breast epithelial cells, and unregulated estrogen signaling can lead to the development of breast cancer. There is extensive evidence for independent roles of AIB1 and BRCA1 in estrogen-stimulated gene expression. AIB1 has a positive effect on estrogen-stimulated proliferation of breast cells whereas BRCA1 has a negative effect. AIB1 increases the sensitivity of breast cancer cells to estrogen and growth factor signaling. We showed that AIB1 is able to partially reverse the suppression of estrogen receptor activity by BRCA1 in human breast cancer cells. This effect does not seem to be through a direct interaction with BRCA1. Epidermal growth factor (EGF) receptor tyrosine kinase family members regulate many oncogenic processes including growth, metastasis, and angiogenesis. It was then determined if AIB1 and BRCA1 affect biological responses, signaling, and gene expression due to activation by heregulin-beta and EGF, which are growth factors of EGF receptor family members. Both AIB1 and growth factor signaling regulate expression of the cyclin D1 gene. As a measurement for cyclin D1 gene expression, there was a greater increase of cyclin D1 promoter activity in MCF-7 (BRCA1 wild-type) breast cancer cells with heregulin-beta than with EGF. In both HCC1937 (BRCA1-deficient) and MCF-7 (BRCA1 wild-type) breast cancer cells, induction of -1745 cyclin D1 promoter activity by heregulin-beta was enhanced with overexpression of AIB1-delta3, which suggests that enhancement of cyclin D1 promoter activity by AIB1-delta3 is independent of BRCA1 status in the cell. A reduction in AIB1 expression did not effect heregulin-beta-induced activation of HER3; however, EGF-induced activation of EGFR was decreased. Therefore, it was determined if AIB1 levels were limiting for EGF-induced proliferation in breast cancer cells. A reduction in AIB1 expression resulted in decreased EGF-stimulated proliferation of MDA-MB-231 (BRCA1 wild-type) breast cancer cells, which was a result of decreased receptor tyrosine phosphorylation and reduced signaling of downstream pathways including mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 5 (STAT5).

In this study, we report a novel observation that cellular AIB1 levels are limiting for EGFR tyrosine phosphorylation, signal transduction, and EGF-stimulated proliferation of breast cancer cells. It still remains to be determined if the BRCA1 status of breast cancer cells affects EGF-induced activation of EGFR. It is possible that AIB1 could regulate BRCA1 function through control of EGFR signaling.

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P32-18: AKT REGULATES BRCA1 STABILITY IN RESPONSE TO ESTROGEN SIGNALING**Andrew Cook Nelson,¹ Traci Lyons,¹ Christian Young,² Steven Anderson,¹ and Jeffrey Holt¹**¹University of Colorado Denver, Health Sciences Center, and ²University of Colorado at Denver

BRCA1 regulates the response to DNA damage in multiple tissues, yet inherited *BRCA1* mutations result specifically in breast and ovarian cancers. Several lines of evidence have suggested a functional relationship exists between steroid hormone signaling and BRCA1 function. Our data demonstrate that estrogen signaling regulates BRCA1 protein expression at a post-translational level. Estrogen stimulation activates the PI3K-AKT pathway leading to AKT-dependent phosphorylation of BRCA1 at S694 and T509. This appears to prevent rapid degradation of BRCA1 by

the proteasome, which is supported by the demonstration that treatment with the clinically utilized proteasome inhibitor bortezomib can similarly lead to a rapid increase in BRCA1 protein levels. AKT activity also supports nuclear localization of BRCA1, and co-expression of AKT and BRCA1 decreases radiation sensitivity suggesting this interaction has functional consequences for BRCA1's role in DNA repair. We conclude that AKT is an important regulator of BRCA1 protein stability and function. The responsiveness of the AKT-BRCA1 regulatory pathway in breast tissue to estrogen signaling may, in part, underlie the tissue specificity of *BRCA1* mutant cancers. Furthermore, pharmacological targets within this pathway could provide strategies for modulation of BRCA1 protein, which may prove therapeutically beneficial for the treatment of breast and ovarian cancers.

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ONCOGENES I

Poster Session P33

P33-1: IN VIVO ROLE OF THE Six1 HOMEOPROTEIN IN MAMMARY GLAND TUMORIGENESIS

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Human Six1 is a homeodomain-containing transcription factor that is critical for cell proliferation, survival, and epithelial-to-mesenchymal transition (EMT) during normal development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression in vivo. We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rTA mice to TetO-Six1 mice and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis in vivo. Low levels of Six1 expression are observed in uninduced transgenic animals over long periods of time, suggesting leakiness in the inducible model. Interestingly, animals treated with doxycycline, as well as uninduced animals, develop marked mammary hyperproliferation and abnormal alveologenesis. In addition, tumor formation is observed after long latency (>1 year) in both induced and uninduced animals, suggesting that low levels of Six1 are sufficient to cause transformation in this model. Tumors formed are complex but are best characterized as invasive ductal adenocarcinomas with complex features. Importantly, sarcomatoid differentiation (spindle cell morphology) is observed, and E-cadherin expression is lost, while the mesenchymal markers Zeb1 and β -catenin are detected in the nuclei of spindle-cell areas of the tumors. Nuclear localization of β -catenin in tumors overexpressing Six1 suggests that the Wnt pathway, a potent mediator of tumorigenesis, may be activated in Six1-driven tumors. Finally, lung metastasis has occurred in a subset of animals. Thus, this transgenic model demonstrates that inappropriate expression of Six1 promotes high-grade tumor formation, oncogenic EMT, and metastasis, suggesting that Six1 is a powerful oncogene that is important not only for tumor initiation but also for tumor progression. Mining of clinical data sets reveals that expression of the Six1 transcriptional complex is an indicator of poor prognosis in a number of different cancers, suggesting that Six1 may play important roles in many different cancer types. As Six1 is not necessary for most normal adult tissues, therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments, making Six1 an attractive chemotherapeutic target.

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P33-2: EPHA2 RECEPTOR TYROSINE KINASE AMPLIFIES ErbB2 SIGNALING, PROMOTING TUMORIGENESIS AND METASTATIC PROGRESSION OF MAMMARY ADENOCARCINOMA

Jin Chen,¹ Dana Brantley-Sieders,¹ Guanglei Zhuang,¹ Donna Hicks,¹ Wei Bin Fang,² Yoonha Hwang,¹ Justin Cates,¹ Karen Coffman,³ Dowdy Jackson,³ Elizabeth Buckerheimer,³ and Rebecca Cook¹

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EphA2 receptor tyrosine kinase overexpression is common in aggressive breast cancer, correlating with a poor prognosis. However, while EphA2 has been reported to enhance tumorigenesis, proliferation, and MAPK activation in some model systems, other reports suggest that EphA2 activation diminishes these processes and inhibits the activity of MAPK upon stimulation. In this study, we deleted EphA2 expression in two transgenic mouse models of mammary carcinoma. EphA2-deficiency impairs tumor initiation and metastatic progression in MMTV-Neu, but not MMTV-PyV-mT, mice. Histologic and ex vivo analyses indicate that EphA2 enhances tumor proliferation and motility. Biochemical analyses reveal that EphA2 forms a complex with ErbB2/Neu human and murine breast carcinoma cells, resulting in enhanced activation of Ras/MAPK and RhoA GTPase. MMTV-Neu, but not MMTV-PyV-mT, tumors were sensitive to anti-EphA2 antibody therapy. Given that both Neu/ErbB2 and polyomavirus middle T antigen activate Ras/MAPK, our data provide evidence that EphA2 represents an important molecular distinction between these two malignant pathways. These data suggest that EphA2 cooperates with Neu/ErbB2 to promote tumor progression and is a novel therapeutic target for ErbB-receptor dependent tumors. Moreover, EphA2 function in tumor progression appears to be dependent upon oncogene context, an important consideration for the application of therapies targeting EphA2.

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P33-3: CYR61 (CCN1) IN HEREGULIN-OVEREXPRESSING BREAST CARCINOMAS: FROM ANGIOGENESIS TO ONCOGENESIS VIA $\alpha_v\beta_3$

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The angiogenic inducer CYR61 (CCN1), a cysteine-rich secreted protein that binds to the extracellular matrix, is differentially overexpressed in breast cancer cells exhibiting high levels of the growth factor Heregulin (HRG), a transactivator of HER2 (*erbB-2*) that closely associates with metastatic breast cancer phenotypes. As a part of our efforts to characterize the ultimate role of CYR61 in breast cancer progression, we have recently demonstrated that CYR61, independently of its upstream regulator HRG, can exhibit oncogenic properties. The ectopic overexpression of CYR61 in the HRG- and HER2-negative MCF-7 cell line, a non-tumorigenic and estrogen-dependent breast cancer in vitro model, was sufficient to promote breast cancer acquisition of hormone independence- and anti-estrogen resistance. Moreover, CYR61 did enhance a metastatic phenotype by promoting cell proliferation and survival in soft agar, cell migration and invasion, Matrigel outgrowth, and tumor formation in immunodeficient mice. Mechanistically, forced expression of CYR61 was found to up-regulate the expression of its own integrin receptor $\alpha_v\beta_3$ (> 200 times), thus generating a CYR61-triggered "CYR61- $\alpha_v\beta_3$ autocrine loop" in the epithelial compartment of breast carcinoma. Down-stream of $\alpha_v\beta_3$, CYR61 overexpression significantly activated the PI-3'K/AKT and MEK/ERK MAPK transduction pathways, both playing a critical role in cell survival and cell proliferation, respectively. In addition, CYR61 overexpression rendered breast cancer cells significantly resistant to Taxol-induced apoptotic cell death. On the other hand, antisense cDNA down-regulation of CYR61 expression abolished anchorage-independent growth, drastically decreased the expression levels of $\alpha_v\beta_3$ and significantly increased Taxol sensitivity in breast cancer cells engineered to overexpress HRG (MCF-7/T clones). Importantly, stable silencing of CYR61 gene by vector-based short hairpin RNA (shRNA) expression systems in MDA-MB-231 breast cancer cells, which constitutively overexpress HRG and CYR61, recapitulated these effects. Thus, when retroviral vectors expressing CYR61-targeted shRNAs from the H1 promoter were used to examine the effects of siRNA knockdown of CYR61 on the transformed properties of the highly metastatic MDA-MB-231 breast cancer cells, the reduced accumulation of CYR61 (up to 90% reduction) caused a dramatic decrease in their colony formation ability in soft-agar assays (up to 95% inhibition), induced the appearance of a fibroblastic morphology and promoted hypersensitivity to Taxol-induced cell damage. These findings not only reveal that CYR61 is intrinsic to the malignant behavior of breast cancer cells but further provide a starting point to evaluate CYR61 as a novel target for the treatment of breast cancer disease using either chemically stable inhibitors of the CYR61-driven cellular signaling (i.e., $\alpha_v\beta_3$ antagonists) or cell-selective vector systems able to RNAi targeting CYR61 gene.

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P33-4: ROLE OF LYSOPHOSPHOLIPIDS IN THE INITIATION, PROGRESSION, AND THERAPY OF BREAST CANCER

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We have demonstrated previously that lysophosphatidic acid (LPA), the simplest phospholipid signaling molecule, is elevated in ascites and plasma of ovarian cancer patients as well as plasma in multiple other cancers. LPA acts through specific G-protein-coupled receptors (GPCRs) mediating a plethora of protumorigenic activities. Others and we have demonstrated that LPA is produced extracellularly from lysophosphatidylcholine by autotaxin (ATX/LysoPLD) that was originally identified as an autocrine motility factor for melanoma cells and is implicated in tumor progression. Both LPA receptors and ATX are aberrantly expressed in several cancers including breast cancer. However, the roles played by LPA receptors or ATX in breast carcinogenesis are poorly understood, especially in vivo. Our specific aims are: (1) to determine whether over-expression of LPA receptors and/or ATX contributes to the initiation of breast cancer and (2) to determine the pathways and mechanisms by which LPA induces tumor formation in mammary glands. To this end we have generated four transgenic mouse models in which LPA receptors (LPA1, LPA2, or LPA3) or ATX are expressed under the control of the mouse mammary tumor virus (MMTV) promoter. Three founders of MMTV-LPA1 and two founders of MMTV-LPA2, MMTV-LPA3, and MMTV-ATX successfully passed the transgene through in every germ line. Breast epithelium-specific expression has been confirmed at the level of RNA, protein, and function in the 4 strains. Strikingly, the expression of ATX or each of LPA1, LPA2, or LPA3 receptors was sufficient to result in high-frequency, late-onset (ages from 8 months to 23 months) mammary carcinomas in multiple lines with variable incidence. In MMTV-LPA 1, 2, and 3 transgenic mice, the incidence of mammary carcinoma development in MMTV-LPA2 is highest (46.51%) with the earliest onset (average 15.6 months); in MMTV-LPA1 transgenic mice, the incidence of mammary carcinoma development is the lowest (32.65%), with an average 18.8 months incubation period; the incidence in MMTV-LPA3 is 44%, between LPA2 and

LPA1, with the longest tumor-free period (average 19.5 months). A total of 51.36% of the MMTV-ATX mice developed mammary carcinoma at an average age of 20.0 months. In contrast, no mammary cancers developed in a cohort of 38 multiparous WT females that were similarly bred, consistent with the reported very low mammary tumor incidence (<1%) in FVB/N mice, suggesting that tumor development is related to LPA receptors or ATX transgene expression rather than random insertional events. In addition, these models result in ER-positive, metastatic tumors, a process that is rarely seen in murine transgenic models. Histopathological analysis of mammary glands and mammary carcinoma shows that chronic mastitis with squamous metaplasia is common lesion in mammary glands from MMTV-LPAs and MMTV-ATX mice suggesting that the tumors could be driven by inflammation. We demonstrate for the first time that LPA plays a causal role in tumorigenesis of the mammary gland in MMTV-LTR-driven mouse models, which provides an improved understanding of breast tumorigenesis as well as potential targets for diagnosis and therapy of breast cancers. Importantly, the tumors are driven by overexpression of wild-type human LPA receptors and the enzyme-producing LPA. Further, since LPA binds to GPCRs and almost one half of all drugs in clinical use target these receptors, validation of LPA as a target in breast cancer could lead to new forms of therapy.

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P33-5: TRANSFORMING PROPERTIES OF 8p11-12 CANDIDATE ONCOGENES IN HUMAN BREAST CANCER

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The 8p11-12 genomic region is amplified in approximately 15% of human breast cancers. Using quantitative genomic PCR and array comparative genomic hybridization (CGH), we have recently mapped the 8p11-12 amplicon in three human breast cancer cell lines: SUM-44, SUM-52, and SUM-225 and in 90 primary human breast cancers. Our detailed genomic analysis results suggested that the 8p11-12 region, which has a similar complex amplification pattern as those observed at 11q13, 17q22-24, and 20q12, may contain more than one important gene. We have further identified 21 candidate genes from the 8p11-12 amplicon based on statistical association between copy number and expression. To elucidate the individual and cooperative oncogenic properties of these genes in human breast cancer, we established a cDNA expression library containing these 21 candidate oncogenes using a lentiviral expression system. We transduced the lentiviral library with individual and different combinations of candidate oncogenes into MCF10A human mammary epithelial cells and selected recipient cells in serum-free medium lacking insulin-like growth factor and/or epidermal growth factor. A total 8 of the 21 genes, WHSC1L1, DDHD2, SPFH2, LSM1, BAG4, TC-1, BRF2, and PROSC, could individually induce insulin-like growth factor-independent growth, but not EGF independence. Additionally, growth curves and colony formation assays in MCF10A cells with equalized viral titer of the tested genes indicated that overexpression of WHSC1L1 most dramatically enhanced cell proliferation in insulin-deficient media. We next investigated each of eight genes for the expression of other transformed phenotypes including growth in soft agar, invasive capacity, and altered morphogenesis in Matrigel. The results of these experiments showed that these eight genes induce different combinations of transforming phenotypes. Five genes, WHSC1L1, DDHD2, LSM1, BAG4, and TC-1, induce anchorage-independent growth in soft agar assays; three genes, WHSC1L1, DDHD2, and SPFH2, have the ability to alter morphogenesis in Matrigel; and three genes, DDHD2, SPFH2, and LSM1, induce invasive capacity. We further measured the activation of insulin receptor substrate-1 (IRS-1) and -2 (IRS-2) to evaluate IGF-1 pathway activity associated with overexpressing these genes in MCF10A cells. Only MCF-10A cells overexpressing DDHD2 and SPFH2 had detectable levels of IRS-1 tyrosine phosphorylation, suggesting that cells overexpressing these genes may grow independently of IGF receptor activation for their insulin-like growth factor-independent growth. However, cells transformed by WHSC1L1, DDHD2, SPFH2, TC-1, and BAG4 showed constitutive phosphorylation of 4E-binding protein 1 (4EBP1). Our data indicate that the 8p11-12 amplicon harbors multiple oncogenes including WHSC1L1, DDHD2, TC-1, and LSM1 with different abilities to induce transformed phenotypes in human mammary epithelial cells.

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P33-6: TUMOR SUPPRESSOR RETINOBLASTOMA PROTEIN (pRb) COOPERATES WITH BASIC HELIX LOOP HELIX (bHLH) MYO D TO MAINTAIN A TERMINAL CELL CYCLE ARREST

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An aberrant and uncontrolled cell proliferation is a characteristic feature of all cancers and may be caused by a number of possible factors including a deregulation of tumor suppressor protein called retinoblastoma protein (pRb). In normal cells, pRb plays a key role in both controlled cell division and terminal differentiation process towards

cellular maturation enabling cells to perform specific functions. Notably, the process of cell differentiation leads cells to an irreversible cell cycle arrested stage, thus preventing a re-entry to the cell cycle. The ability of pRb to control such terminally arrested stage categorizes it as a tumor suppressor protein. Intriguingly in normal cells the pRb function depends upon its ability to switch between phosphorylated and dephosphorylated stages, which is eventually controlled by another protein called cyclin D1 and its kinase partner cdk4. Motivated by the earlier published research work from our lab and the observations from other investigators, I started working with the hypothesis that pRb may be controlling the expression of cyclin D1. And the mechanism of regulating cyclin D1 is by an indirect manner through control of one or more immediate early genes. Since pRb has been implicated in playing an important role in terminal differentiation of skeletal muscle, thus I have used this process as my working model to dissect role of pRb in terminal cell cycle arrest. Using a pRb defined genetic background fibroblast turned myoblasts (either positive or negative for pRb expression), I have shown for the first time that a master regulator of skeletal muscles differentiation, MyoD cooperates with pRb to control the expression of immediate early genes and eventually cyclin D1, thus maintains a terminally arrested stage.

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P33-7: THE LIM-ONLY FACTOR LMO4 REGULATES EXPRESSION OF THE BMP7 GENE THROUGH AN HDAC2-DEPENDENT MECHANISM, AND CONTROLS CELL PROLIFERATION, AND APOPTOSIS OF MAMMARY EPITHELIAL CELLS

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The nuclear LIM-only protein LMO4 is upregulated in breast cancer, especially estrogen receptor-negative tumors, and its overexpression in mice lead to hyperplasia and tumor formation. Here, we show that deletion of LMO4 in the mammary glands of mice leads to impaired lobuloalveolar development due to decreased epithelial cell proliferation. Engrailed-LMO4 fusion protein also decreases proliferation and increases apoptosis of normal human mammary epithelial cells. With the goal of discovering potential LMO4-target genes, we developed a conditional expression system in MCF-7 cells for both LMO4 and a dominant negative (DN) form of its co-regulator, Co-factor of LIM domains (Clim/Ldb/Nli). We then used DNA microarrays to identify genes responsive to LMO4 and DN-Clim upregulation. One of the genes common to both datasets was BMP7 whose expression is also significantly correlated with LMO4 transcript levels in a large dataset of human breast cancers suggesting that BMP7 is a bona fide target gene of LMO4 in breast cancer. Inhibition of BMP7 partially blocks the effects of LMO4 on apoptosis indicating that BMP7 mediates at least some of functions of LMO4. Gene transfer studies show that LMO4 regulates the BMP7 promoter, and chromatin immunoprecipitation studies show that LMO4 and its co-factor Clim2 are recruited to the BMP7 promoter. Furthermore, we demonstrate that HDAC2 recruitment to the BMP7 promoter is inhibited by upregulation of LMO4 and that HDAC2 knockdown upregulates the promoter. These studies suggest a novel mechanism of action for LMO4: LMO4, Clim2 and HDAC2 are part of a transcriptional complex, and increased LMO4 levels can disrupt the complex, leading to decreased HDAC2 recruitment and increased promoter activity.

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P33-8: THE ROLE OF TRANSFORMING GROWTH FACTOR- β SIGNALING IN Six1-INDUCED EMT AND METASTASIS

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Background: During embryogenesis, the homeodomain transcription factor Six1 is critical in the development of multiple organs where it directs precursor cell proliferation, cell survival, and epithelial-to-mesenchymal transitions (EMT). In most normal adult tissues including the mammary gland, Six1 is not expressed, however, in human breast cancer it is re-expressed in 50% of primary and 90% of metastatic lesions and significantly correlates with shortened time to metastasis and to relapse, and with decreased disease-specific survival. Recent work in our lab demonstrates that Six1 expression in immortalized, but non-tumorigenic mammary epithelial cells induce properties of EMT and lead to aggressive tumor formation in an orthotopic model of breast cancer. Together, these data implicate Six1 in tumor progression. Based on Six1's

ability to induce EMT during development and in cancer, we investigated the role of the transforming growth factor- β (TGF- β) pathway that is highly correlated with both EMT and metastasis in Six1-induced tumor progression.

Objective: The objective of this research is to identify potential mechanisms of Six1-induced tumorigenesis and progression in mammary epithelial cells including the modulation of the TGF- β pathway by Six1.

Results: The expression of Six1 in both the immortalized mammary epithelial cell line, MCF12A, and the mammary carcinoma cell line, MCF7, increases expression of transforming growth factor- β receptor I (T β RI). Increased expression of T β RI in mammary epithelial cells correlates with increased TGF- β signaling as measured by phosphorylation and nuclear localization of Smad3 and increased basal and TGF- β -induced expression of the TGF- β -responsive reporter, 3TP-luciferase. Activation of the TGF- β pathway in response to Six1 begs the question whether TGF- β signaling underlies Six1's role in tumor progression and particularly in the induction EMT-like properties. TGF- β is a known inducer of EMT, a process that is correlated with increasing malignant potential in cancer. Indeed, Six1 expression in mammary epithelial cells induces properties of EMT including downregulation or relocation of E-cadherin, increased β -catenin transcriptional activity, alterations in cell-matrix adhesion, and increased invasion. More importantly, Six1 expression in the non-metastatic MCF7 cell line induces lymphatic and bone metastases after orthotopic injection into the mammary glands of nude mice, consistent with TGF- β 's pro-metastatic role in other models of breast cancer. Based on the established role of TGF- β in EMT and metastasis particularly to the bone, we hypothesize that Six1 contributes to increased tumor malignancy and metastasis through upregulation of TGF- β signaling.

Conclusions: The expression of the Six1 homeobox protein in mammary epithelial cell lines increases expression of T β RI and correlates with increased TGF- β signaling that suggests that TGF- β signaling may underlie Six1's ability to induce EMT and metastatic spread. Understanding the mechanisms by which Six1 induces breast cancer formation and progression has the potential to direct therapeutic strategies and also reinforces the concept of Six1 as a good therapeutic target in breast cancer.

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P33-9: STRUCTURE-FUNCTION ANALYSIS OF THE Six1 TRANSCRIPTIONAL COMPLEX

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Cancer and normal development share many properties. During normal development, genes are activated that stimulate the proliferation of progenitor cells, as well as migration, invasion, angiogenesis, and they can also alter cell survival. These genes are often down-regulated once organ development is complete. In cancer, many developmental genes are re-activated, stimulating the aforementioned processes out of context. The homeobox gene Six1 encodes a transcription factor that plays a critical role in the proliferation, survival, and migration of cells during the development of many organs and is also implicated in the progression of a number of different types of tumors, including breast carcinomas. Six1 is amplified in human breast cancer and its mRNA is overexpressed in 50% of primary breast tumors and 90% of metastatic lesions. Six1 overexpression has been linked to enhanced cellular proliferation, transformation, and increased tumor volume in breast cancers. Interestingly, in breast cancer Six1 overexpression correlates with metastasis. Out of more than 130 breast cancers examined, Six1 overexpression correlates significantly with positive lymph node status ($p < 0.05$). Most importantly, reduction of Six1 levels using RNA interference leads to statistically significant decreases in tumor cell proliferation and metastasis in vivo, strongly suggesting that Six1 will make an excellent cancer therapy target. Because Six1 is expressed during embryogenesis, lost in most differentiated adult tissues, and re-expressed in tumors, we believe it is an ideal drug target whose inactivation will inhibit tumor cell proliferation and metastasis with limited side effects. The purpose of this work is to lay the foundation for developing tumor-specific chemotherapeutic agents for a prevalent women's cancer, as well as additional cancers in which Six1 has been implicated. This foundation will be generated by using x-ray crystallography to determine the 3-dimensional structure of the Six1 transcriptional complex. Using the resulting structure, we will be able to use an innovative rational drug design approach against a novel transcriptional complex (Six1/Eya) involved in the progression of breast and many other carcinomas; a complex that has never before been clinically targeted but is expected to inhibit both tumor cell proliferation and metastasis.

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P33-10: MOLECULAR PROFILING OF BREAST CANCER CELL LINES DEFINES RELEVANT TUMOR MODELS AND PROVIDES A RESOURCE FOR CANCER GENE DISCOVERY

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Introduction: Breast cancer cell lines have been used widely to investigate breast cancer pathobiology and new therapies. Breast cancer is a molecularly heterogeneous disease, and it is important to understand how well and which cell lines best model that diversity. In particular, microarray studies have identified molecular subtypes – luminal A, luminal B, ErbB2-associated, basal-like and normal-like – with characteristic gene-expression patterns and underlying DNA copy number alterations (CNAs). Here, we studied a collection of breast cancer cell lines to catalog molecular profiles and to assess their relation to breast cancer subtypes.

Methods: Whole-genome DNA microarrays were used to profile gene expression and DNA copy number alteration (CNA) in a collection of 50 widely used breast cancer cell lines, and comparisons were made to existing profiles of primary breast tumors. Hierarchical clustering was used to identify gene-expression subtypes, and Gene Set Enrichment Analysis to discover biological features of those subtypes. CNAs were analyzed to identify co-varying loci and subtype-specific associations. Genomic and transcriptional profiles were integrated to discover candidate cancer genes with extreme CNA and altered expression.

Results: Transcriptional profiling of breast cancer cell lines identified a luminal and two basal (A and B) subtypes. Luminal lines displayed an estrogen receptor (ER) signature and resembled luminal-A/B tumors, basal-A lines were associated with ETS-pathway and BRCA1 signatures and resembled basal-like tumors, basal-B lines displayed mesenchymal and stem-cell characteristics, and ErbB2+ lines straddled both luminal and basal subtypes. Compared to tumors, cell lines exhibited similar loci of CNA and co-varying CNAs, but an overall higher complexity of CNA (genetically simple luminal-A tumors were not represented), and little conservation of subtype-specific CNAs. Fifty-one focal DNA amplifications and 18 deletions, with concomitantly altered gene-expression, identified known and novel candidate breast cancer genes.

Conclusions: Overall, breast cancer cell lines were genetically more complex than tumors but retained expression patterns with relevance to the luminal-basal subtype distinction. The compendium of molecular profiles defines cell lines suitable for investigations of subtype-specific pathobiology, biomarkers, and therapies, and provides a resource for discovery of new breast cancer genes.

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P33-11: OVEREXPRESSION OF CrkII LEADS TO ABNORMAL MAMMARY GLAND DEVELOPMENT AND BREAST CANCER

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The v-Crk protein was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus. Cellular homologues of v-Crk, include c-Crk, which encodes two alternatively spliced proteins (c-CrkI and c-CrkII) and c-CrkL. The Crk adaptor proteins are composed of one SH2 and one or two SH3 domains and play an important role during cellular signaling by mediating the formation of protein-protein complexes involved in cellular migration, invasion, and adhesion. We have previously shown that the overexpression of Crk proteins promotes dispersal of organized epithelial cells and converts a noninvasive EGF response to an invasive one, implicating a role for Crk proteins in cancer. This has important implications as we have found elevated levels of Crk in human breast cancer. However, the question of whether Crk adaptor proteins can lead to breast cancer development has not been addressed. Thus, we created a transgenic mouse model that allows the overexpression of CrkII through the hormonally responsive MMTV promoter. In each CrkII line studied, virgin mice undergoing puberty (7-10 weeks) were found to have delayed ductal outgrowth, compared to their wild-type FVB littermates. This indicates a slowed development of the mammary gland and was characterized by increased collagen surrounding the terminal end bud. No significant changes were found in proliferation, apoptosis, or differentiation within these structures. The fat pad was eventually filled and in mice at 20 weeks of age, there was precocious ductal branching. The ductal branching was associated with increased PCNA staining, suggesting enhanced proliferation. Tumor development as well as the formation of hyperplasias were monitored in both virgin and multiparous females to determine if CrkII is a potent oncogene in breast cancer. Focal mammary tumors or hyperplasias subsequently appeared in 35% of the transgenic animals studied with an average latency of 14 months. Overexpression of CrkII led to the formation of spindle cell carcinoma, which may be induced by an epithelial-to-mesenchymal transition (EMT). This is interesting, as our lab has previously demonstrated that Crk proteins can induce EMT-like changes in vitro. Another CrkII tumor was identified as squamous adenocarcinoma, whose pathology is similar to

MMTV models in which the Wnt pathway is activated. Staining was positive for CK14+ (myoepithelial marker), CK6+ (a putative progenitor marker), and CK8+ (luminal marker) suggesting that these tumors are of mixed lineage. These results highlight a specific role for CrkII in breast cancer development, as other adaptor and scaffold proteins over-expressed in the mammary gland are unable to induce tumorigenesis (i.e., MMTV-Grb2, MMTV-p130Cas, MMTV-Gab2). Thus, the present study demonstrates that the CrkII adaptor protein plays an important role in integrating signals for mammary gland development and breast cancer progression.

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P33-12: MAMMARY GLAND TUMOR DEVELOPMENT IN TRANSGENIC MICE OVEREXPRESSING DIFFERENT ISOFORMS OF THE CUX1 TRANSCRIPTION FACTOR

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The CUX1 transcription factor is involved in several processes including cell cycle progression, cell migration and invasion, and the determination of cell-type identity. In nontransformed cells, the full-length protein of 200 kDa (p200 CUX1) is proteolytically processed by nuclear cathepsin L at the G1/S transition of the cell cycle into an isoform of 110 kDa (p110 CUX1). A second isoform of 75 kDa (p75 CUX1) is generated from an mRNA that is initiated within intron 20. Many studies suggest that the short CUX1 isoforms p75 and p110 are involved in cancer development. The p110 and p75 isoforms are overexpressed in primary human tumors, such as in uterine leiomyomas and breast cancers. Also, proteolytic processing of CUX1 is increased in many transformed cells and is no longer cell cycle regulated. Furthermore, in tissue culture, p110 and p75 stimulate cell proliferation by accelerating entry into S phase. To investigate the oncogenic potential of CUX1, we engineered transgenic mice overexpressing p75, p110, or p200 under the control of the mouse mammary tumor virus promoter (MMTV). Each transgene was specifically integrated into the hypoxanthine phosphoribosyltransferase (hprt) locus and then backcrossed into the FVB background. Interestingly, in the three lines, 40% to 50% of mice developed tumors by the age of 24 months, which is significantly above the wild-type level of 19%. Tumors arose mainly in the mammary gland, the uterus, and the lungs. Mammary gland tumors in the three lines were highly heterogeneous, with some being solid carcinomas, others containing areas of squamous metaplasia or papillary differentiation, and others being more glandular. In many tumors, the transgene was found to be overexpressed and activated. Experiments performed with cell lines established from the CUX1 tumors revealed that inhibition of CUX1 reduced the migration capacity of these cells. Tumors from the p200 CUX1 transgenic mice were found to overexpress cathepsin L and p110 CUX1 and to display enhanced p110 activity. These findings suggest that increased proteolytic processing of p200 CUX1 could drive tumor development in this mouse line. Finally, from ChIP-on-chip analyses in the Hs578t cell line, several Wnt genes were identified as putative targets of CUX1, and some of these, such as wnt1 and wnt10A, were found to be overexpressed in the CUX1 mammary tumors. In summary, this study provides evidence that CUX1 can act as an oncogene in breast epithelial cells and emphasizes the need to study and decipher the mechanisms of action of this transcription factor.

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P33-13: EXPRESSION OF STAT1 REGULATES ErbB2-INDUCED TRANSFORMATION AND TUMORIGENESIS

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The signal transducer and activators of transcription (Stat) are a family of cytoplasmic proteins with roles as signal messengers and transcription factors that participate in cellular responses to cytokines and growth factors. The first member of the family to be identified, Stat1, plays a role in many cellular processes including development, cell growth, proliferation, and apoptotic cell death. The best-characterized function of Stat1 is its role in the cellular response to interferon (IFN) and virus infection. Stat1 knockout mice are extremely susceptible to infection with viruses and other pathogens demonstrating the essential role of this transcription factor in innate immunity. The cellular role of Stat1 is not limited to combating viral infection; numerous tumor suppressor functions of Stat1 have also been identified. Specifically, Stat1 knockout mice exhibit increased susceptibility to chemical carcinogens relative to their wild-type counterparts. Also, mating of Stat1^{-/-} with p53^{-/-} mice yields animals with a higher tumor incidence and a broader spectrum of tumors than p53^{-/-} mice. Stat1 also mediates inhibition of cell proliferation and an induction of apoptosis in response to treatment with IFN-gamma or tumor necrosis factor alpha (TNF-alpha), possibly via its ability

to upregulate caspases and the cyclin-dependent kinase (cdk) inhibitor p21. Recent work in our lab has demonstrated that Stat1 inhibits transformation by oncogenic Ras in a manner dependent upon the site-specific phosphorylation of Stat1. In our current study, we set out to determine if Stat1 exerted the same inhibitory effect in ErbB2-induced breast tumorigenesis.

To examine the potential effect of Stat1 in mediating ErbB2 transformation, p53^{-/-}/Stat1^{-/-} mouse embryonic fibroblasts were made to stably overexpress a transforming mutant of ErbB2. When transplanted into nude mice, these cells formed large tumors. These cells were then reconstituted with wild-type and phosphorylation mutant forms of Stat1 to assess the effect on ErbB2 function. Using this system, we demonstrated that expression of Stat1 substantially reduces the development of transformed characteristics in ErbB2-expressing cells in vitro as well as alters cellular proliferation rates. Interestingly, the ability of Stat1 to impede anchorage-independent growth appears independent of phosphorylation status, which is contrary to the effect observed in Ras-transformed cells. To complement the results of the in vitro experiments, we examined the effect of Stat1 expression in a mouse model of breast cancer. When ErbB2 transgenic mice were crossed onto a Stat1^{-/-} background, preliminary results indicated that development of breast tumors was more rapid when Stat1 expression was reduced (i.e., in a heterogeneous Stat1 background).

These results suggest a previously unknown function of Stat1 in modulating the development of ErbB2-induced transformation in vitro and the development of breast tumors in vivo. Given the fact that overexpression of ErbB2 in breast tumors is associated with poor prognosis and increased risk of metastasis, the effects of Stat1 in regulating ErbB2-induced transformation may be exploited to develop new targeted therapies.

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P33-14: IMMORTALIZATION AND TRANSFORMATION OF HUMAN MAMMARY EPITHELIAL CELLS BY c-Myc AND c-Myc PHOSPHORYLATION DEFICIENT MUTANTS

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The c-Myc transcription factor is commonly dysregulated in breast cancer. c-Myc has been shown to regulate a number of genes, including human telomerase reverse transcriptase (hTERT), the catalytic subunit of the enzyme telomerase. Telomerase activity is characteristic of most immortalized and transformed cells, and immortalization is considered an early event in cancer development. The c-Myc N-terminal domain contains several phosphorylation sites, including threonine-58 (T58) and serine-62 (S62). Previous studies have utilized rodent fibroblast systems to show an impact of altering phosphorylation at these two sites on protein stability, apoptosis, and transformation. In this study, we compared the ability of wild-type c-Myc or c-Myc phosphorylation deficient mutants—c-Myc T58A, S62A, and T58A/S62A—to immortalize and transform human mammary epithelial cells (HMECs). We used retroviral transductions to create stable cell lines overexpressing the various c-Myc constructs. The c-Myc phosphorylation deficient mutants appear to be more efficient at promoting cellular immortalization than wild-type c-Myc. All c-Myc constructs were functional in HMECs as evidenced by their ability to induce hTERT transcription as seen with real-time PCR and telomerase activity as measured by quantitative telomeric repeat amplification protocol (TRAP) assay. The immortalized HMEC lines exhibited near-diploid karyotypes with specific chromosomal abnormalities, suggesting clonality. Most importantly, the immortalized lines overexpressing c-Myc T58A (and to lesser extent wild-type c-Myc) acquired anchorage-independent growth after further cell passaging. The ability of c-Myc T58A HMECs to form colonies in soft agar was accompanied by a unique clustering morphology that included detachment from the tissue culture substrate and free-floating growth in the media. When HMECs previously immortalized with hTERT were transduced with c-Myc T58A, limited growth in soft agar was seen, indicating that c-Myc T58A expression played a partial role in anchorage-independent growth. Finally, we identified a number of genes that were differentially expressed between c-Myc T58A HMECs before and after acquiring the ability for anchorage-independent growth via gene array analysis. Our findings suggest that differences in c-Myc protein phosphorylation can impact c-Myc biological activity in human breast epithelial cells. In addition, these cell lines provide a unique tool for identifying the molecular and genetic changes during transition from the immortal to anchorage-independent states.

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P33-15: THE ROLE OF p38 IN SIGNALING EVENTS DOWNSTREAM OF ONCOGENIC Ras**Lynne Waldman and Robert Weinberg**
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p38 is a mitogen-activated protein kinase (MAPK) that is strongly activated by inflammatory cytokines and environmental stresses including osmotic shock, oxidative stress, and ionizing radiation. Furthermore, p38 signaling regulates many cellular processes, such as cell cycle arrest, apoptosis, and differentiation. The ability of p38 to promote these antiproliferative cellular processes suggests that p38 could possess tumor suppressive functions. In support of this idea, a p38-null background in mouse embryonic fibroblasts (MEFs) was found to promote their transformation by oncogenic Ras. Also, p38 has been found to facilitate Ras-induced senescence in human fibroblasts. The objective of the work that will be presented at the Era of Hope 2008 Meeting is to better understand the role of p38 in the signaling events that are downstream of oncogenic Ras. Both the signaling events that cause p38 to be activated in response to oncogenic Ras and the functional outcomes of these p38-dependent signaling events will be presented.

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P33-16: AIB1-Δ3 IS A MORE ONCOGENIC ISOFORM OF THE NUCLEAR RECEPTOR COACTIVATOR AIB1**Christopher Chien**
Lombardi Comprehensive Cancer Center

Amplified In Breast Cancer 1 (AIB1) is a member of the p160 steroid receptor coactivator family. AIB1 and its corresponding family members have a normal function to coactivate transcription of nuclear receptor responsive target genes by recruiting molecules involved in chromatin remodeling, which facilitates the assembly of general transcription factors. However, AIB1 has been found to be amplified in a number of breast cancer cell lines and primary breast tumor samples and has been defined as an oncogene. The AIB1 gene was found to be amplified in 5%–10% and protein overexpressed in 50%–90% of breast tumors. Activation of AIB1 is responsible for enhancing pro-growth/anti-apoptotic genes, which are involved in breast cancer carcinogenesis. Independent of hormone, AIB1 can promote tumor growth through other transcription factors such as E2F1, AP-1, NF-κB, which are required for growth factor signaling (IGF-1, EGFR, and HER-2). We have previously identified a splice variant of AIB1, AIB1-Δ3, that is highly expressed in a number of breast cancer cell lines and tumor samples and is a more potent coactivator than AIB1. The ability of AIB1-Δ3 to be a more potent coactivator is puzzling since AIB1-Δ3 lacks the N-terminal 198 amino acids of AIB1, which contains the nuclear localization sequence of AIB1. In this study, we define the stability, cellular localization and trafficking, and protein interactions of AIB1-Δ3, which distinguish it from AIB1. Interestingly, we found that AIB1-Δ3 is more resistant to proteasomal degradation in high density and serum withdrawal conditions. AIB1 is located primarily in the nucleus while AIB1-Δ3 is primarily in the cytoplasm, but AIB1-Δ3 is shown to accumulate in the nucleus upon nuclear export blockade suggesting that AIB1-Δ3 is imported into the nucleus. Since AIB1-Δ3 is a more potent coactivator than AIB1 we tried to determine if differences in protein interactions were responsible for the contrast in stability and cellular localization of the two isoforms. We find that AIB1-Δ3 is imported into the nucleus by binding to full length AIB1, which provides a nuclear localization signal in trans. We are currently investigating the possibility of the N-terminal 198 amino acids of AIB1 to contain a domain for binding an inhibitory protein that does not bind to AIB1-Δ3. Given the above data, we see that AIB1-Δ3 is a more potent coactivator because of its increased protein stability, cellular trafficking, and protein interactions, which makes it a more important factor in oncogenesis.

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P33-17: Y1226 MEDIATES ErbB2 INDUCED PROTECTION FROM APOPTOSIS**Alexandra Lucs and Senthil Muthuswamy**
Cold Spring Harbor Laboratory

ErbB2/Her2/Neu is a receptor tyrosine kinase that is overexpressed in 25%–30% of breast cancers. This overexpression correlates with poor clinical prognosis in node-positive patients. ErbB2 transforms cells by promoting proliferation, by disrupting

epithelial architecture, and by inhibiting apoptosis. To determine if ErbB2 controls these phenotypes through pathways emanating from specific or multiple autophosphorylation sites, we created a series of autophosphorylation site mutations where one of five autophosphorylated tyrosines was left intact while the other four were mutated to phenylalanine. In our studies we examined ErbB2 mutants retaining Y1144, Y1201, Y1226/7, and Y1253.

The mutated receptors were expressed in a nontransformed human mammary epithelial cell line, MCF-10A. MCF-10A cells grown on a laminin and collagen-rich matrix form three-dimensional structures similar to acini found in the breast. As these three-dimensional structures mature, they become organized with a hollow central lumen, and the cells undergo proliferation arrest. Activation of ErbB2 in mature structures re-initiates cellular proliferation, induces lumen filling, and disrupts normal acinar organization, creating multiacinar structures. Thus, the 3D acini structures allow us to study all three of the ErbB2-induced phenotypes in a single system.

We have tested the autophosphorylation site mutations in this assay and found that both the increase in proliferation and the disruption of acinar organization were induced by activation of all the ErbB2 mutants. In contrast, full inhibition of apoptosis was only observed after activation of the ErbB2 construct retaining Y1226/7.

Overexpression of ErbB2 in cell lines sensitive to chemotherapeutic drugs, such as paclitaxel (Taxol®), results in increased cell survival. Furthermore, patients with ErbB2-positive tumors respond to Taxol treatment better when combined with the anti-ErbB2 antibody Herceptin®. We show that activation of the ErbB2 construct retaining Y1226/7, but no other site, protects cells from Taxol-induced apoptosis. Thus, our results demonstrate that while ErbB2 induces proliferation by activating pathways downstream of multiple autophosphorylated tyrosine residues, it inhibits apoptosis by activating pathways downstream of Y1226/7. Our current investigation is aimed at identifying the pathway(s) downstream of Y1226/7 that is responsible for ErbB2-mediated protection of Taxol-induced apoptosis.

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P33-18: REGULATION OF SENESENCE BY p38 MAP KINASE IN ErbB-2-INDUCED BREAST CANCER**Carola Neumann, Lauren Ball, and Scott T. Eblen**
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Oncogene-induced senescence is a cellular response that may be crucial for protection against cancer development. Senescence in vivo is found in precancerous tissue as an attempt to arrest growth of tumor-prone cells, whereas the “escape” from senescence represents tumor initiation. Oncogene-induced senescence can be triggered by ERB-2 or Ras through the increase of intracellular reactive oxygen species (ROS) and the activation of p38 MAP kinase. However, it has not been determined which substrates of p38 are utilized in vivo to initiate or terminate tumor suppressive cellular senescence.

We have shown that mice lacking the antioxidant protein peroxiredoxin 1 (Prdx1) die prematurely due to hemolytic anemia and cancer. Prdx1^{-/-} MEFs have higher levels of ROS and oxidative induced DNA-damage. They demonstrate slower proliferation with an increased portion of G1 phase cells, suggesting a senescence-like phenotype. These cells also have a higher susceptibility to oncogenic transformation by ErbB2 or Ras as a result of the loss of Prdx1 antioxidant activity. The breast cancer model MMTV-RasV12 mice demonstrate greatly decreased median breast cancer survival when crossing with prdx1^{-/-} mice. Basal and serum-stimulated p38 activity is higher in prdx1^{-/-} MEFs compared to wild-type, suggesting that loss of Prdx1 could activate p38 senescence signaling. p38 binds to its substrates targets through docking site domains on both the kinase and the substrate. We have previously demonstrated that a single point mutation in the ATP binding site of MAP Kinases allows them to uniquely utilize analogs of ATP, allowing for the specific labeling and identification of direct substrates in a complex mixture of proteins.

The objective of this proposal is to identify novel and known p38 MAP Kinase substrates that regulate oncogene-induced senescence and how substrate utilization changes during the transition to proliferation and tumorigenesis. We hypothesize that substrate utilization by p38 changes throughout oncogene-induced tumorigenesis and that differential substrate utilization regulates these transitions. By identifying pro-senescence, pro-proliferative, and pro-apoptotic p38 substrates in primary mammary epithelium, we hypothesize that future therapeutic agents can be generated that drive preferential utilization of pro-apoptotic p38 substrates, thus eliminating oncogenic cells.

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STROMAL- EPITHELIAL INTERACTIONS I

Poster Session P34

P34-1: ARE MESENCHYMAL STEM CELLS THE ORIGIN OF CARCINOMA-ASSOCIATED FIBROBLASTS?

Izhak Haviv
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In animal models of prostate, lung, ovarian, and breast cancers, normal epithelial cells can become malignant when surrounded by fibroblasts that are derived from tumors (carcinoma-associated fibroblasts). We therefore seek the molecular basis of this phenotypic carcinoma promotion of fibroblasts within cancer tissue. In spite of the robust reaction of fibroblasts and epithelial cells to co-culture, in our hands, fibroblasts from normal reduction mammaplasty fail to turn into cancer-promoting fibroblasts via co-cultivation in xenografts with cancer cells. Furthermore, fibroblast genome was found to be intact in 21 of 22 tumor-juxtaposed fibroblasts obtained through microdissection of ovarian and breast cancer sections. We used microarray analysis of defined co-cultures to identify intercellular signals that contribute to cross-talk between epithelial cells and fibroblasts. A reciprocal interaction that drives an inflammatory gene expression signature in fibroblasts was observed. A related signature of genes was found to be tightly co-expressed in human cancer expression profiles. Additional genes in this signature from human expression profiles reflect infiltrates of inflammatory cells that respond to the fibroblast-derived chemokines, particularly CCL2, CCL5, and CCL8. A comparative semi-supervised analysis based on clinical outcome confirmed that the expression of this gene signature is exhibited in a group of patients with inherently poor prognosis, due to elevated metastatic potential, and microdissection of tissue sections showed that indeed these genes are in fact expressed in tumor-juxtaposed fibroblasts. Using chimeric mice, in which mesenchymal precursors (MP) are beta-Gal labeled, as hosts for mammary tumor cell line in a syngeneic model, we demonstrate that MP infiltrate primary tumors. Effective recruitment of the MP to primary tumors, to assist in tumor cell dissemination and/or the establishment of metastatic sites, depends on specific genes, such as CCL5, but the recruiting signal from the cancer remains elusive. This work offers a potential source for this recruitment, that is, the residential fibroblast transient response to tissue injury and interaction with the cancer cell. In addition, this work offers a compendium of experimental growth conditions and their consequential gene expression changes and assesses which of those are most robust and consistent in a large fraction of cancer patients of a variety of carcinomas. The resultant interaction-expression associations may focus future attempts to modulate the cancer microenvironment.

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P34-2: THE ORIGINS AND POTENTIAL FUNCTIONS OF MYOFIBROBLASTS IN BREAST CARCINOMAS

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Phenotypically many human carcinomas, especially those of the breast, colon, and pancreas, display a stroma that is distinct from that found in the normal tissue and the stroma often comprises a majority of the tumor mass. The tumor stroma is a collection of various cell types found intermingled with the neoplastic epithelial cells and often includes fibroblasts, myofibroblasts, endothelial cells, and immune cells. In most carcinomas, myofibroblasts are the most prevalent stromal cell type. Additionally, myofibroblasts have been demonstrated by several groups to play an important role in tumor progression. However, little is known about the source of myofibroblasts within carcinomas. Therefore, the objective of this study is (1) to determine if a cell type within the peripheral blood, the peripheral blood fibrocyte (PBF), is recruited to sites of tumor formation where it gives rise to a myofibroblast and (2) to delineate the contribution of fibrocytes to tumor progression. To identify the source of recruited stromal cells, xenograft tumors were generated in Nude mice reconstituted with Rag1-/- GFP transgenic bone marrow. I have characterized the stromal cells recruited from the blood to the BPLER breast carcinoma model through immunohistochemistry and flow cytometry analysis and demonstrated that some of the cells express markers that are found on fibrocytes including: CD34, CD45, CD11b, and Collagen I. A portion of the stromal cells recruited from the blood express smooth muscle actin (SMA), a marker of differentiated fibrocytes and myofibroblasts. Additionally, preliminary results show that a majority of the cells recruited from the blood express both CD45 and CD11b. When the cells recruited from the blood to the tumor are compared with those that are recruited to the lung, preliminary results reveal a CD11b+ bone marrow-derived cell type is preferentially recruited to the site of BPLER tumor formation. The information from these experiments suggests that fibrocytes are recruited to sites of carcinoma progression, but their contribution to tumorigenesis requires further experimentation. In conclusion, if we know specific cell types in the blood are recruited to the tumor stroma, then new therapeutics can be designed that disrupt this recruitment and thereby help to inhibit tumor growth. Furthermore, identification of a cell type that homes preferentially to the site of a tumor could potentially provide a novel method for delivering drugs to the sites of cancer formation.

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P34-3: THE IMPACT OF ADIPOSE STROMAL CELLS ON THE BEHAVIORS OF BREAST CANCER CELLS

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Epidemiological studies have identified obesity as a significant risk factor for developing breast cancer among postmenopausal women. Adipose tissue is an important extragonadal source of estrogen. Although circulatory estrogen levels in postmenopausal women decline, tissue-specific estrogen levels remain virtually unchanged and are even found elevated in breast tissue from obese patients and patients with breast cancer. Therefore, excessive estrogen production due to overall adiposity is considered to be a critical contributing factor to obesity-associated breast cancer risk. Indeed, using adipocyte stromal cells (ASCs) isolated from lipoaspirates from cancer-free patients, we showed that ASCs in general have a great inducibility of aromatase expression. Furthermore, there appears to be a huge individual-based variability in aromatase transcription. In a parallel study, we also examined the influence of ASCs on tumor cell behaviors. Using the Boyden-chamber assay, we found that ASCs had a stimulatory effect on the migration of both ER-positive (MCF-7) and ER-negative breast cancer cells (MDA-MB-231), suggesting that ASCs can influence the behaviors of breast cancer cells in an estrogen-independent manner. A similar effect of ASCs on MDA-MB-231 cells was also observed in a wound-healing assay. Furthermore, in a xenograft experiment using co-transplanted MDA-MB-231 and ASCs, we observed a significant role of ASCs in promoting the invasiveness of MDA-MB-231 cells. These data indicate that, in addition to their established role in supplying local estrogen, ASCs may facilitate tumor invasion into surrounding tissue and subsequently metastasis. The possible molecular basis for this effect of ASCs on tumor cell migration and invasion will be discussed.

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P34-4: THE ROLE OF CXCL12 AND CXCL14 CHEMOKINES IN EPITHELIAL STROMAL CELL INTERACTIONS DURING BREAST TUMOR PROGRESSION

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Increasing evidence suggests that changes in the cellular microenvironment contribute to tumorigenesis, but the molecular basis of these alterations is not well understood. We have characterized molecular alterations that occur during breast tumor progression in each cell type that compose normal breast tissue and in situ (DCIS-ductal carcinoma in situ) and invasive breast carcinomas using SAGE (serial analysis of gene expression) for gene expression profiling, SNP (single nucleotide polymorphism) arrays and array CGH (comprehensive genomic hybridization) for analyzing genetic changes, and MSDK (methylation specific digital karyotyping) for the characterization of DNA methylation profiles. Using these approaches, we determined that gene expression and epigenetic changes occur in all cell types during breast tumor progression while clonally selected genetic alterations are limited to tumor epithelial cells. We also determined that dramatic changes occur in the normal to DCIS transition while we were not able to identify distinct in situ and invasive carcinoma molecular signatures. These findings suggest that the progression of in situ to invasive carcinoma may be influenced by non-epithelial cells. Correlating with this hypothesis, a significant fraction of abnormally expressed genes encode secreted proteins and receptors implicating a role for abnormal autocrine/paracrine signaling in breast tumor progression. Among others, the CXCL14 and CXCL12 chemokines overexpressed in tumor myoepithelial cells and myofibroblasts, respectively, bind to receptors on epithelial cells and enhance their proliferation, migration, and invasion. Thus, chemokines may play a role in breast tumorigenesis by acting as paracrine factors.

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P34-5: VITRONECTIN IN THE TUMOR MICRO-ENVIRONMENT PROMOTES BREAST CANCER CELL PROLIFERATION THROUGH ELEVATED PROTEIN SYNTHESIS BY INTEGRIN $\alpha_v\beta_3$ ACTIVATION OF THE mTOR 4E-BP1 PATHWAY

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Background: It is well established that the extracellular matrix (ECM) modulates critical tumor cell processes including survival, differentiation and angiogenesis. We asked whether the ECM also regulates protein synthesis in tumor cells. During hypoxia, tumor cell protein synthesis is generally down-regulated, impairing tumor growth and proliferation. The regulation of translation by integrins through the Akt/mTOR/4E-BP1 pathway represents a pivotal process that impacts on tumor cell responses to hypoxia, invasion and progression. We demonstrate that the integrin $\alpha_v\beta_3$, which is often elevated on breast tumor cells and promotes migration and inva-

sion, interacts with vitronectin, an ECM component only available to tumors with invasive disruption of the basement membrane, resulting in sustained and high levels of protein synthesis and tumor cell proliferation despite hypoxia, through the constitutive activation of the mTOR/4E-BP1 pathway.

Material & Methods: Breast cancer cell lines were tested for overall protein synthesis rates and VEGF-specific mRNA translation under conditions of normoxia and hypoxia (1% O₂, 24 hours). Cells were cultured on ECM components: native or denatured collagen IV, fibronectin, laminin, vitronectin. Protein synthesis rates were determined by 35S-methionine specific activity incorporation into protein. Levels of VEGF were determined by ELISA. Antibody LM609 against the integrin $\alpha_5\beta_3$ was used in blocking experiments.

Results & Discussion: Under normoxia, overall protein synthesis levels and VEGF expression were unaffected by growth on different ECM components. Surprisingly, whereas most breast cancer cell lines decrease protein synthesis and proliferation under hypoxia, MDA-MB-435 breast cancer cells strongly increased overall protein synthesis and proliferation (>2 fold), but only when grown in contact with vitronectin. Vitronectin becomes an ECM component during tumor invasion and metalloproteinase activity that disrupts the barrier basement membrane, and is associated with increased tumor migration, proliferation, and invasion. MDA-MB-435 breast cancer cells express high levels of the integrin $\alpha_5\beta_3$, which also is associated with increased breast cancer cell migration and invasion, and engages vitronectin. To determine whether $\alpha_5\beta_3$ is responsible for conferring vitronectin-mediated enhanced tumor cell protein synthesis and tumor cell proliferation under hypoxia, cells were cultivated on vitronectin in the presence of control or anti- $\alpha_5\beta_3$ antibodies. Blocking of $\alpha_5\beta_3$ eliminated enhanced protein synthesis and migration of MDA-MB-435 cells under hypoxia but not VEGF expression. Analysis of the Akt/mTOR/4E-BP1 signal transduction pathway, which is stimulated by integrin engagement and controls protein synthesis, demonstrated that vitronectin interaction with $\alpha_5\beta_3$ maintained stimulation of Akt, mTOR and inactivation of translation repressor 4E-BP1 despite hypoxia, thereby sustaining elevated protein synthesis required for tumor cell proliferation.

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P34-6: MCF-7 BREAST CANCER CELLS DOWN-REGULATE MACROPHAGE MIGRATION INHIBITORY FACTOR IN CO-CULTURED MACROPHAGES: IMPLICATIONS FOR CONTEXT-SPECIFIC MODULATION OF TUMOR-ASSOCIATED MACROPHAGES

Theodore A. Bremner, Giselle T. Burnett, Denise C. Weathersby, and Tiffany E. Taylor
Howard University

Inflammatory cells in the tumor microenvironment support tumor progression by secreting inflammatory cytokines and growth factors that promote angiogenesis, invasion, and metastasis. Key modifier genes of tumor progression have been found to be expressed in tumor-associated macrophages (TAMs), rather than in transformed epithelium. Consequently, it has been suggested that pharmacological modification of TAMs may be an effective therapeutic strategy. Because of the importance of macrophages in innate immunity, total ablation of macrophages would be undesirable; whereas, context-specific targeting of TAMs should be more effective. A fuller understanding of stromal cell-cancer cell interactions is necessary to identify phenotypic changes in macrophages that may render them selectively vulnerable to pharmacological agents. We used gene arrays, reverse transcription-polymerase chain reaction, and Western blot analysis to study inflammation- and angiogenesis-related gene expression in co-cultured MCF-7 cells and THP-1-derived macrophages, and to determine how interactions between the two cell types are affected by tamoxifen and aspirin.

Our results show that macrophages and MCF-7 cells express macrophage migration inhibitory factor (MIF) when grown separately; however, in co-culture, MIF expression was up-regulated in cancer cells, but dramatically down-regulated in macrophages. Surprisingly, tamoxifen-pretreated MCF-7 cells up-regulated MIF in co-cultured macrophages. Two molecular variants of MIF were identified: a "macrophage form" and an "MCF-7 form." The macrophage form was present in co-cultured MCF-7 cells, whereas, the MCF-7 form was not found in co-cultured macrophages. MIF is a pleiotropic cytokine, with pro-inflammatory and angiogenic properties, that plays a direct role in normal cell division and oncogenic cell transformation by promoting survival via the Akt pathway. As an upstream activator of macrophages, MIF enhances expression of genes encoding tumor necrosis factor- α , interleukin (IL)-1, IL-6, and IL-8, and is a determinant of the M1 (killing) macrophage phenotype. The MCF-7-induced ablation of MIF expression in TAMs may be associated with a partial M2 (healing) polarization of these cells. We show that MCF-7 cells do not express IL-10 when grown separately or in co-culture, and that aspirin induced IL-10 expression in macrophages and in MCF-7. However, aspirin-pretreated macrophages potentially induced IL-10 expression in MCF-7 cells, suggesting that aspirin treatment can imprint macrophages to induce IL-10 expression in MCF-7 cells. Moreover, aspirin induced IL-10 expression in tamoxifen-pretreated MCF-7 cells, suggesting that

tamoxifen can modulate the MCF-7 response to aspirin. Our results indicate that reciprocal regulation of gene expression in cancer cells and TAMs can produce dynamic tumor phenotypes that may be rational targets for new anticancer therapies. While MIF upregulation in MCF-7 may be adaptive, MIF downregulation in TAMs may be protective for tumor cells, but may confer on TAMs a context-specific vulnerability to agents that abrogate MIF-mediated anti-apoptosis. Finally, the ability of tamoxifen-pretreated MCF-7 cells to upregulate MIF expression in co-cultured macrophages may represent a previously unrecognized effect of tamoxifen in inhibiting tumor progression.

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P34-7: TGF β 1 ACTS AS A MAMMARY TUMOR PROMOTER IN THE IRRADIATED HOST

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Current paradigms for cancer initiation and progression generally focus on epithelial cell mutations although it is increasingly clear that the development of cancer is highly intertwined with the host microenvironment. We have shown that ionizing radiation induces persistent phenotypic changes in the mouse mammary stroma that are in part mediated by activation of transforming growth factor β 1 (TGF β 1). We developed a stromal-epithelial mammary chimera model to determine whether the irradiated host affects mammary tumor development. This model takes advantage of the fact that the mammary epithelium can be removed from prepubertal mammary glands and the parenchyma-free stroma can then serve as the recipient of transplanted mammary epithelium. We transplanted *Trp53* null mammary tissue, which recapitulates important features of human breast cancer, e.g., long latency, ductal carcinoma in situ lesions, estrogen receptor positivity. To examine whether TGF β 1 contributes to tumor promotion by host irradiation, syngeneic *Trp53* null mouse mammary fragments were transplanted to Balb/c wild-type (WT) and *Tgfb1* heterozygote (HT) mice. Inguinal glands of hosts were cleared of epithelium at 3 weeks of age and the mice irradiated whole body with 10, 50, and 100 cGy 60Co γ -radiation at 12 weeks of age. *Trp53* null mammary tissue from 10–12 week old donors was transplanted 3 days later. In WT hosts, 73% of *Trp53* null mammary transplants developed carcinomas at approximately a year of age compared to 58% in *Tgfb1* HT hosts. The mean latency increased from 250 days in WT to 300 days in HT hosts. Histopathology revealed a pronounced difference between tumors from WT and HT hosts. WT hosts were predominantly adenocarcinomas while HT hosts were mostly spindle cell carcinomas. Consistent with this, 60% of *Tgfb1* HT tumors were α -smooth muscle actin positive compared to 30% of tumors from WT hosts. Thus host genotype is an important determinant of cancer progression. Furthermore, tumor frequency increased to 100% in nearly all irradiated WT hosts although it did not affect latency or tumor histology. Importantly, the effect of host irradiation on tumor

frequency was abrogated in *Tgfb1* HT hosts. The data in this model support the hypothesis that the action of carcinogens is promoted via effects on the microenvironment, and that in the case of radiation, that this is in large part mediated by TGF β 1.

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P34-8: FUNCTIONS OF PTEN IN STROMAL FIBROBLASTS DURING DEVELOPMENT OF MAMMARY EPITHELIAL TUMOR

Jean-Leon Chong and Anthony J. Trimboli
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Different types of cells within the tumor microenvironment have been suggested to significantly influence initiation, progression, and metastasis. Fibroblasts are the major cellular component of tumor environment and play a key role in the synthesis of growth factors, chemokines, and most other constituents of the extracellular matrix. Alterations in *Pten* have been described in melanoma, brain cancer, and prostate cancer. Significantly, close to 30%–50% of patients with breast cancer will either have a mutation or loss of one copy of the *PTEN* gene. Previous work revealed that loss of *Pten* in the stroma of invasive breast cancers occurred at a surprisingly high frequency. The function affected by the stroma-specific mutation remains to be elucidated. This raises an intriguing question of how *PTEN* affects mammary gland tumorigenesis. To address how *PTEN* protein functions in tumor stroma of mammary gland, our lab has generated a conditional allele of *Pten* (*PtenLoxP*) and developed transgenic mice that express the *cre* recombinase under control of *Fibroblast-specific protein* (*Fsp1*) promoter in stromal fibroblasts of the mammary gland (*Fsp-cre*). By breeding *PtenLoxP/LoxP* and *Fsp-cre* with *MMTV-ErbB2* mice, we have investigated how loss of *Pten* in stromal fibroblasts affects mammary epithelial cancers.

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P34-9: CONJUGATED LINOLEIC ACID EFFECTS ON ADIPOSE-EPITHELIAL SIGNALING IN BREAST CANCER

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Conjugated linoleic acids (CLA) have been extensively studied as inhibitors of carcinogenesis; however, clear mechanisms of action of these dietary fatty acids remain elusive. The most reproducible effects of CLA have been the consistent changes in body fat content induced by the *trans*-10, *cis*-12 isomer (10e12z). Adipose is positively correlated with breast cancer and, as one of the predominant stromal cell types in the mammary gland, actively regulates the growth of the epithelium. Therefore, we asked whether the reduction in adipose is related to 10e12z CLA's described effects in carcinogenesis. To address this question, we are examining gene expression changes in the 3T3-L1 adipocyte model in response to 10e12z CLA to identify adipocyte-derived factors that may modulate the growth of the mammary epithelium. Using a microarray based approach; treatment of preadipocytes with 50 μ M 10e12z CLA for 12 hours altered expression of few genes but significantly induced extracellular matrix (ECM) molecules. However, treatment of fully differentiated adipocytes for 12 hours with 50 μ M 10e12z CLA strongly repressed expression of adipose markers including C/EBP α , and PGC-1 α while inducing many inflammatory markers (NF- κ B, IL-6, MCP-1 and 2) as well as ECM regulators. Because COX-2 is an important inflammatory mediator and is regulated by NF- κ B, we analyzed its expression by real-time PCR. Cox-2 expression is induced by CLA by day 5 during differentiation of preadipocytes treated with 10e12z CLA, consistent with changes in markers of adipose function and gene expression. In adipocytes, COX-2 expression is rapidly induced to a maximum level by 12 hours after treatment and is significantly induced by as little as 12.5 μ M 10e12z CLA. Together these results indicate that adipocytes are more responsive to 10e12z CLA than preadipocytes and that COX-2 may mediate some of the effects of 10e12z CLA on body fat content. However, these results also indicate that 10e12z CLA may induce a reactive stroma condition that is inconsistent with a protective role in breast cancer. These results suggest that further clarification of the mechanism of 10e12z CLA action is required before recommendations can be made about its use in breast cancer prevention. We are currently exploring whether the changes in adipocytes induced by 10e12z CLA modulate the growth of the mammary epithelium.

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P34-10: AGED STROMA AND TUMORIGENESIS

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Cellular transformation and the subsequent development of a neoplasia are the result of a stepwise process in which an incipient cancer cell evolves from a normal cell into a fully transformed cell capable of invasion and metastasis. While it is clear that these cell autonomous changes are integral to the transformation process, it has become evident that changes in the surrounding, ostensibly normal stroma are also critical. Indeed, nontumorigenic fibroblasts within a tumor secrete factors that promote tumor cell growth. Similarly, senescent fibroblasts promote tumor growth in xenograft models. Indeed, we have identified senescent stroma in premalignant lesions suggestive of its potential role in an endogenously arising tumor. To understand how senescent fibroblasts promote preneoplastic cell growth, we undertook a microarray approach and investigated the expression of genes uniquely modulated in senescent fibroblasts. Our analysis has revealed factors that may be critical for senescent fibroblast-prenoplastic cell interaction. Utilizing viral-based RNAi, we have begun to elucidate the role of these candidates in our system and have uncovered potential signaling pathways important for such heterotypic interactions. Given that genetic mutations and the presence of senescent cells increase with age, these studies may shed light on why a significant increase in tumor incidences is observed in older individuals. In addition, these studies may uncover novel antineoplastic targets directed to the tumor stroma.

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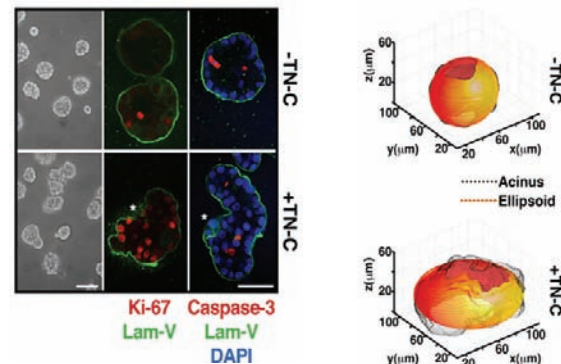
P34-11: STROMAL TENASCIN-C PROMOTES EPITHELIAL CELL PROLIFERATION TO DISRUPT NORMAL MAMMARY TISSUE ARCHITECTURE AT THE LUMINAL LEVEL

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Induction of tenascin-C (TN-C), a stromally-derived extracellular matrix (ECM) glycoprotein, is associated with breast cancer development and progression, yet its role in this disease remains obscure. To investigate the effects of TN-C on normal human mammary epithelium, MCF-10A cells were cultivated in a three-dimensional

(3-D) reconstituted basement membrane (BM), either with or without exogenous TN-C. Whereas under control conditions cells formed polarized acinar structures with a continuous BM and a central lumen (resulting from site-specific apoptosis), exposure to TN-C provoked loss of BM and increased cell proliferation without affecting apoptosis. To determine how these changes alter mammary epithelial tissue structure and function, an active contours-based algorithm was developed that allowed us to generate 3-D renditions of mammary acini, which were then used to quantify acinar topography and volume. Although TN-C increased acinar surface roughness, it did not affect volume. Based on these results, we hypothesized that TN-C promotes epithelial cell proliferation within the lumen, and that this process may involve c-met, a receptor tyrosine kinase which is overexpressed in breast tumors, where it is believed to affect both lumen formation and cell proliferation. Indeed, TN-C-treated acini contained filled lumens and expressed higher levels of c-met than controls. Inhibition of c-met in the presence of TN-C resulted in phenotypic reversion of acinar architecture. In addition, breast cancer tissue enveloped by a TN-C-rich stroma expressed higher levels of epithelial c-met when compared to normal tissue. Collectively, these 3-D studies indicate that TN-C compromises mammary epithelial tissue homeostasis via its effects on BM integrity, c-met expression, and luminal epithelial cell proliferation, making it a promising therapeutic target in breast cancer.



Stromal TN-C alters normal 3-D mammary epithelial tissue architecture

TN-C increases acinar surface roughness

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P34-12: BIOLOGICAL FUNCTION FOR PLASMA KALLIKREIN IN MAMMARY GLAND INVOLUTION

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The plasminogen cascade of serine proteases has been affiliated with both development and tumorigenesis in the mammary gland. The ultimate effector in this cascade, plasminogen (active form: plasmin), is managed by a complicated cascade of plasminogen activators and protease inhibitors. This study focuses on the role of the plasminogen cascade of serine proteases in the last stage of murine mammary development: involution. Plasminogen can be activated to plasmin by urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and plasma kallikrein (PKal). Though tPA and uPA are efficient activators of plasminogen, mice deficient for either or both of these plasminogen activators do not recapitulate the mammary gland involution phenotype of plasminogen null mice. Instead, the dominant plasminogen activator for mammary stromal involution is PKal (zymogen = prekallikrein), a serine protease that participates in the contact activation system of blood coagulation that is present in high concentration (40 μ g/ml) in circulating plasma. A variant of ecotin, a macromolecular inhibitor of the trypsin family of serine proteases derived from *E. coli*, was engineered to be highly specific for the active PKal protease. We have shown this ecotin specific for PKal (ecotin PKal) delays alveolar apoptosis, adipocyte replenishment, and stromal remodeling in the involuting mammary gland resembling the phenotype of plasminogen-deficient mice. This effect on involution is relieved 5 days after the end of ecotin PKal treatment. Furthermore, using a tagged form of ecotin PKal, we have visualized active PKal in vivo. As such, PKal has been localized to connective tissue-type mast cells in the stroma and surrounding the blood vessels of the murine mammary gland and does not appear in tissues devoid of connective tissue-type mast cells or tissues containing mucosal-type mast cells. Additionally, the specific localization of PKal to mast cells is dependent on the activation state of the mast cells. Lastly, we have shown that plasma kallikrein expression is not limited to liver hepatocytes as previously thought; RT-PCR on RNA collected from mammary glands at different developmental stages revealed that PKal is expressed highly during times of stromal remodeling such as during puberty or postlactational involution.

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P34-13: THE ROLE OF CELL TRACTION FORCE IN FIBRONECTIN FIBRILLOGENESIS**Lewis Romer,¹ Christopher S. Chen,² and Christopher A. Lemmon¹**¹Johns Hopkins University School of Medicine and ²University of Pennsylvania

The biochemical and mechanical properties of the extracellular matrix environment influence the growth behavior of mammary epithelia and may alter the behavior of breast cancer. In a reciprocal fashion, breast cancer cells may alter the character of the matrix through paracrine effects on mammary fibroblasts. In the current work, we simultaneously measure fibronectin fibrillogenesis, cell spreading, and cell-generated traction forces in order to define the role that cell traction forces play in matrix assembly. This was done by plating NIH 3T3 cells that constitutively express YFP-labeled fibronectin onto microfabricated post array detectors (mPADs). mPADs consist of a uniform array of deformable PDMS cantilever posts that were microcontact-printed with fibronectin. These provide discrete contact points for each individual site of cell-matrix adhesion. Traction forces were calculated by measuring deflections of the

posts while immunofluorescence imaging was used to visualize fibronectin and quantify fibrillogenesis. Cells were plated for up to 48 hours, and samples were fixed and imaged at time intervals from 3 to 48 hours to collect data on a large cohort of cells. In addition, individual cells were tracked in a live-cell imaging assay. We demonstrate that fibronectin fibrillogenesis can be predicted from the map of traction forces generated by the cell. Specifically, we show that the direction of fibronectin assembly is determined by the vector of cell traction force applied at the fibril's point of origin. Further, fibril length and thickness are determined by the ratio of force applied beneath the interior of the cell to force applied at the cell edge. These data indicate that fibronectin fibrillogenesis is directed by the distribution and direction of traction forces exerted by fibroblasts at the cell-matrix interface. Understanding the mechanisms by which breast epithelia and fibroblasts interact to change the mechanics of the matrix microenvironment may lead to novel approaches to breast cancer treatment.

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TUMOR PROGRESSION I

Poster Session P35

P35-1: EPIGENETIC REGULATION OF BREAST CANCER PROGRESSION

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Transforming growth factor-beta (TGF- β)-Smad signaling pathways play critical but opposing roles in both tumor suppression and metastatic cancer promotion, an early tumor suppressive and a late-stage pro-oncogenic role concomitant with a progressive increase in locally secreted TGF- β levels. It has also been shown that high incidence of breast cancer metastasis is associated with an increase in phosphorylated Smad2 in the nucleus. These observations suggest that the TGF β -TGF β R-Smad2 signaling axis is involved in breast cancer metastasis and it is a legitimate target for therapeutic intervention. One of the clues supporting this rationale is the fact that there was a significant degree of suppression of the in vivo incidence of macrometastases in mouse models for breast cancer upon overexpression of the inhibitory Smad7. As a critical step to studying the effect of Smad7 on invasiveness and metastasis of breast cancer, we characterized model cell lines by analyzing the expression patterns of various candidate epithelial/mesenchymal markers and the basal activity of TGF- β /Smad signaling pathway. Our studies show that the loss of E-cadherin and down-regulation of gamma-catenin expression in cell lines representing the advanced stages are likely to be primarily due to epigenetic alterations derived from differential DNA hypermethylation of their respective promoter regions. Interestingly, the stable retroviral overexpression of WT-Smad7 showed a profound reversion in the gene expression pattern in these cells accompanied by up-regulation of epithelial markers E-cadherin, gamma-catenin. These studies provide the first direct evidence for the role of aberrant signaling events in epigenetic regulation of gene function in breast cancer. Furthermore, elucidation of the effects of hyperactive TGF- β signaling in breast cancer metastasis may reveal novel gene targets that are amenable for therapeutic applications. A discussion on our recent data to elucidate the molecular basis of this novel connection between epigenetic regulation of genes and advanced breast cancer progression will be presented.

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P35-2: THE CELLULAR LOCALIZATION OF IGFBP5 DETERMINES ITS ONCOGENIC FUNCTIONS IN BREAST CANCER

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Metastasis is a major problem in breast cancer, and the molecular mechanism is not well understood. Using cDNA microarray and tissue microarray technologies, other groups as well as our group found that insulin-like growth factor binding protein 5 (IGFBP5) expression is associated with a poor prognosis and metastasis in breast cancer patients (Van't Veer et al., 2002; Hao et al., 2004; Li et al., 2007). The mechanism by which IGFBP5 promotes metastasis and hence a poor outcome is unknown. A major difficulty in understanding the function of IGFBP5 is the paradoxical observation that ectopic overexpression of IGFBP5 in breast cancer cell lines resulted in a suppressed proliferation. A more detailed analysis of IGFBP5 in breast cancer tissues and in transfected breast cancer cell lines showed that IGFBP5 is located in different compartments in the cells. In cancer tissues, IGFBP5 is in the cytoplasm; in the transfected cell IGFBP5 is in the nucleus. Thus, we hypothesize that localization of IGFBP5 determines its functional outcome of the host cells. We generated a mutant IGFBP5 with deletion of nuclear localization sequence by site-directed mutagenesis and transfected the mutant IGFBP5 into the MDA-MB-435 breast cancer cell line to establish stable clones. We observed that deletions of nuclear localization sequence in IGFBP5 abolished nuclear accumulation and enhanced cytoplasmic localization of IGFBP5. Functional analysis showed that the deletion mutant had a significantly higher ability in cell migration and invasion than parental and vector control. Thus, we demonstrated that specific deletion of nuclear localization sequence in IGFBP5 rendered location of IGFBP5 in the cytoplasm and resulted in increased migration and invasion of breast cancer cells.

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P35-3: INTEGRATING FUNCTIONAL AND STRUCTURAL CANCER GENOMICS TO IDENTIFY BREAST CANCER DRIVERS

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Nearly all epithelial malignancies exhibit numerous karyotypic abnormalities. This complexity of genome structure hampers efforts to identify relevant mutations important for cancer development. However, the development of several complementary experimental approaches now provides the tools necessary to systematically deconvolute somatic cancer genetics. We have initiated a large-scale project to identify and validate breast cancer targets in a comprehensive, genome-wide manner. By systematically integrating data from three approaches (high-throughput RNA interference screens, whole-genome analysis of copy number alterations, and cell-based transformation studies), we have identified new oncogenes including IKBKE, which activates the NF- κ B pathway in breast cancer. Using this approach, we have identified other genes involved in breast cancer initiation and progression. Together, this integrated systematic approach permits the rapid identification and validation of new breast cancer drug targets with high promise for clinical translation.

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P35-4: THE HEDGEHOG PATHWAY REGULATES OSTEOPONTIN EXPRESSION IN BREAST CANCER CELLS

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The roles of the Hedgehog (Hh) signaling pathway and osteopontin (OPN) in normal cellular development are well established. Several studies independently implicate both the Hh pathway and OPN in tumor development and metastasis. The Hh pathway is triggered by the binding of the Hh ligand to the 12-pass transmembrane receptor, Patched (Ptc) that catalytically reverses the inhibition of the 7-pass transmembrane protein Smoothened (Smo) resulting in signal transduction that causes the nuclear translocation of the transcription factors, Gli1 or Gli2 or Gli3, culminating in regulation of target gene expression. Deregulation of the Hh pathway has been reported in breast cancer. Expression OPN, specifically within tumor cells, reciprocally correlates with patient survival. Clinically, plasma levels of OPN correlate with tumor burden and metastasis in patients with breast cancer metastasis. We have previously reported that the expression of OPN by breast cancer cells parallels their aggressive phenotype.

Here we report that breast cancer cells express members of the Hh pathway viz. the transmembrane proteins, Ptc and Smo, the transcription factors Gli1 and Gli2, and the ligands Ihh and Shh. Moreover, Gli1 is constitutively nuclear in the human, metastatic breast cancer cells, implying that the Hh pathway is constitutively active. Treatment of breast cancer cells with the Hh ligands Shh and Ihh boosts signaling via the Hh pathway and upregulates the promoter activity of OPN. Conversely, treatment of these cells using cyclopamine, an inhibitor of the Hh pathway, blocked the nuclear translocation of Gli1 and markedly decreased OPN expression. Treatment with cyclopamine also caused a significant reduction in the reporter activity from the OPN promoter, suggesting that the Hh pathway transcriptionally regulates OPN. We next sought to determine if the Hh signaling was mediated by the transcription factor Gli1. While exogenous expression of Gli1 stimulated OPN transcription, siRNA knockdown of Gli1 inhibited OPN.

Thus, we have established that OPN is a target of Hh signaling in breast cancer. Examination of the OPN promoter ~1.8kb upstream of the transcription start site revealed 5 putative Gli-binding sites. Studies are under way to further unravel the role of the Hh pathway in regulation of OPN in breast cancer.

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P35-5: ESE-1 IS REQUIRED TO MAINTAIN THE TRANSFORMED PHENOTYPE OF MCF-7 HUMAN MAMMARY EPITHELIAL CELLS

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Background and Objectives: ETS factors comprise a large transcription factor family known to play a significant role in cellular development, differentiation, and transformation. The ETS transcription factor family is known to play a significant role in many cancers, with aberrant expression of the human Epithelial Specific Ets factor-1,

ESE-1, being correlated in nearly 50% of early human breast tumors. We have previously shown that ectopically expressed ESE-1 imparts the transformed phenotype on MCF-12A and MCF-10A nontransformed mammary epithelial cell lines. Furthermore, dominant-negative ETS approaches have also reversed the transformed phenotype in several breast cancer cell lines (NmuMG, MMT, and BT20). However, such dominant-negative approaches interfere with multiple ETS factors. Thus, the role of any single, specific ETS factor in mammary tumorigenesis remains unknown. In this study, we determine the protein expression of the ETS protein, ESE-1, and address the specific role of a single ETS factor, ESE-1, in maintaining the transformed phenotype in human breast cancer cell lines.

Methods: We developed a highly specific anti-ESE-1 mouse monoclonal antibody and used it in western blot and indirect immunofluorescent cytochemical analysis to determine ESE-1 protein expression and its subcellular localization. We characterized ESE-1 protein expression in MCF-7, T47D, ZR-75, and MDA-MB-231 transformed, tumorigenic mammary epithelial cell lines, and nontransformed MCF-10A and MCF-12A cells. We also generated shRNAs targeting ESE-1 and determined whether ESE-1 knock-down affected the transformed state of MCF-7 cells. Finally, we addressed mechanism by measuring apoptosis and cell proliferation in ESE-1 knock-down cells.

Results: We show that ESE-1 is expressed as a nuclear protein in MCF-7, T47D and ZR-75 transformed, tumorigenic mammary epithelial cell lines, but ESE-1 was not detected in transformed MDA-MB-231 and nontransformed MCF-10A and MCF-12A cells. We also show that the knock-down of a single ETS factor, ESE-1, reverses the transformed phenotype in MCF-7 breast cancer cells. Importantly, we show that ESE-1 is required to maintain the transformed phenotype in MCF-7 breast cancer cells, since shRNA-mediated ablation of endogenous ESE-1 protein resulted in decreased colony formation and anchorage-independent growth. Furthermore, mechanistic studies, using two separate approaches to measure apoptosis and proliferation, revealed that ESE-1 does not modulate apoptosis but rather is required for cancer cell proliferation. Finally, we also demonstrate that serum is required for ESE-1 protein production, and since ESE-1 is required for MCF-7 breast cancer cell proliferation, then ESE-1 must be a key effector mediating cell proliferation.

Conclusions: Taken together, these data contribute novel insights to our understanding of the critical role of ESE-1 in maintaining cell transformation of mammary epithelial cells and that it does so via regulation of cellular proliferation.

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P35-6: ChIP-DSL TECHNOLOGY REVEALS AN EXTENSIVE SLUG-BINDING PROGRAM ON HUMAN GENE PROMOTERS IN BREAST CELLS

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ChIP-DSL, a ChIP technique coupled with microarray provides a powerful tool to determine in vivo binding profiling of transcription factors to deduce regulatory circuitries in mammalian cells. We profiled SLUG-regulated genes in human breast cells using a full human genome promoter array based on the ChIP-DSL technology (Aviva Systems Biology, San Diego), revealing many SLUG target genes. Two human breast cells, MCF7 and MDA-MB-468, do not express any detectable levels of SLUG mRNA and protein. We expressed C-terminal 3xFLAG-tagged SLUG in these cells for ChIP-DSL analysis. The ORF of human SLUG was PCR amplified from the cDNA made from RNA isolated from MDA-MB-231 cells and cloned in frame into the MCS of the p3xFLAG vector (Sigma). The stop codon of the ORF was deleted so that the 3xFLAG epitope can be tagged at the C-terminal of the recombinant protein. For inducible expression of 3xFLAG-tagged SLUG, we employed the ViraPower T-Rex Lentiviral Expression System (Invitrogen). The 3xFLAG-tagged SLUG cDNA insert was amplified by PCR and was subcloned into the pLenti4/TO/V5-DEST vector (Invitrogen). The resulting plasmid was transfected into 293FT cells (Invitrogen) with LipofectAMINE 2000, and an aliquot of the supernatant harvested 3 days after transfection was then added to TetR-expressing MCF7 or MDA-MB-468 cells. The cells were cultured in the presence of blasticidin and zeocin, and the surviving cells that expressed appropriate levels of 3xFLAG-SLUG protein were used in subsequent studies. Expression of 3xFLAG-SLUG in the transformed cells was induced with doxycycline. We performed chromatin immunoprecipitation (ChIP) analysis using the standard protocol supplied by Upstate Biotechnology with their ChIP assay reagents. For immunoprecipitation, we used commercially available antibody against the 3xFLAG epitope (Sigma). ChIP DNA directed selection and primer ligation was done following the reagents and protocols supplied by Aviva Systems Biology. Using these probes we screened a 20K human promoter microarray (Aviva Systems Biology). Out of 20,000 promoters screened, 3xFLAG-SLUG was found to bind significantly and reproducibly with 153 gene promoters in both SLUG over expressing MCF7 and MDA-MB-468 cells. Further analysis of these data and independent follow up validation experiments revealed SLUG as a master regulator for the expressions of several members of the claudin family of tight junction proteins, several key DNA damage response genes including P53 and PALB2, several members of the integrin super family and apolipoprotein B mRNA editing enzyme catalytic polypep-

tide-like 3 (APOBEC3) isozymes in human breast cells. These data will not only be useful to better understand the biology of the SLUG repressor protein in human breast cells but also help in designing molecular decoys in abrogating the function of SLUG during breast cancer cell metastasis.

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P35-7: Rac1 AND Rac1b CONTROL DISTINCT SIGNALING EVENTS IN BREAST CARCINOMA CELLS

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Background: Breast carcinoma cell invasion and metastasis are the main cause of treatment failure for breast cancer. Members of the Rac subfamily of small GTPases, including Rac1 and Rac3, have been implicated in the invasive behavior of a wide range of tumor cells and Rac3 has been found to be constitutively activated in a number of different breast carcinoma cell lines. In related studies, we have shown that Rac1 is critical for invasion in all relatively undifferentiated breast carcinoma cell lines that we have examined to date, whereas Rac3 contributes to the invasive behavior of only some of these cell lines (Chan et al., 2005, *Oncogene* 24: 7821) and Coniglio et al., unpublished observations). Rac1b is a constitutively active splice form of Rac1 that differs from the main splice form of Rac1 by an insert of 19 codons and is expressed in breast tissue. Rac1b is induced during malignant transformation of mammary epithelial cells and causes oxidative damage to DNA and genomic instability (Radisky DC et al., 2005, *Nature* 436: 123). We therefore are examining the roles of Rac1b in the malignant properties of breast carcinoma cells.

Methods: Specific depletion of Rac1b was accomplished by transient transfection of siRNA oligos. We have generated 4 distinct oligos that target the 57 base pair insert region. We also have attempted to generate oligos that specifically target the main Rac1 splice form, without depleting Rac1b, but have not succeeded in this yet in a satisfactory fashion. Cell proliferation was examined using the colorimetric SRB assay and cell invasion was determined using Matrigel-coated transwell chambers. The activation state of signaling molecules was determined using western blotting with phospho-specific antibodies. Most of the functional analysis of Rac1b was performed using ZR75-1 cells, a relatively well-differentiated breast carcinoma cell line. The invasive behavior of these cells is significantly stimulated by heregulin.

Results: Depletion of Rac1 together with Rac1b significantly inhibits cell proliferation, whereas depletion of Rac1b alone has a marginal effect. On the other hand, our preliminary data suggest that specific depletion of Rac1b inhibits heregulin-stimulated invasion of ZR75-1 cells, whereas depletion of both Rac1 and Rac1b enhances invasion in these cells. These surprising results may be accounted for, at least in part, by the observation that inhibiting both isoforms strongly interferes with cell-cell junction formation in ZR75-1 cells, whereas specific depletion of Rac1b does not. Depletion of Rac1b also inhibits heregulin-induced lamellipodia to the same extent as depletion of Rac1b together with Rac1, indicating a critical role for Rac1b in the formation of these structures. Moreover, depletion of Rac1b inhibits the levels of activated ERK and JNK while inhibiting both Rac1b and Rac1 stimulates these kinases, indicating that Rac1 and Rac1b have opposite effects on the ERK and JNK pathways.

Conclusions: Our functional analysis indicates that Rac1 and Rac1b largely control distinct functions pertaining to the malignant transformation of breast carcinoma cells. Thus, specific targeting of the Rac1b splice form may constitute a novel therapeutic strategy to interfere with breast cancer metastasis.

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P35-8: FUNCTIONAL ANALYSIS OF INDIVIDUAL CELLS AND MICROENVIRONMENT OF BREAST CANCER DRAINING LYMPH NODES

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The development of distant metastases is the major cause of death in breast cancer (BC) patients. In many BC cases, metastases are present in patients with no metastasis-positive lymph nodes (LN). Hence, there is a need to improve prognosis by a better prediction of the nodal status and tumor spread. The current study is designed to develop and utilize new functional characteristics of the cells and microenvironment of BC-draining lymph nodes, which may help to improve the estimation of lymph node metastatic involvement. Innovative devices and methodologies were developed for collecting, transferring, and analysis of lymph nodes at an individual cell resolution. Using these devices, a suspension of living cells can be prepared from the lymph node and processed for various assays, including immunophenotypic analysis, activation status, and invasion activity. The methodologies used here provided a heterogeneous selection of living cells, representative of the lymph node cell composition.

They permit manipulation of a limited amount of experimental material, enabling more procedures with the same lymph node, without impairing either the original node structure or the traditional pathological analysis. The functional profile of tumor-activated LN cells showed an increase in the intracellular enzymatic reaction rate, accompanied by a homogeneous distribution of transferrin receptor as well as by a significant increase in matrix metalloproteinases (MMPs) proteolytic activity. Moreover, the proportion of cells exhibiting such a profile was significantly higher in tumor-containing LNs than in tumor free nodes. Thus, the live- and postfixation features of LN cells and their microenvironment, correlated with the functional status of the lymph nodes, which may serve to improve their predictive value in breast cancer examination.

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P35-9: ABSTRACT WITHDRAWN

P35-10: HAPLOINSUFFICIENCY FOR p190B RHOGAP DELAYS MMTV-Neu TUMORIGENESIS

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The Rho signaling pathway regulates many cellular processes including proliferation, survival, migration, mitosis, and cytokinesis. The Rho pathway has also been shown to be important in the development of many types of cancer. p190B RhoGAP works as an inhibitor to regulate the small G-proteins that make up the Rho family. Additionally, lack of p190B has been shown to disrupt embryonic and postnatal mammary gland morphogenesis, and overexpression of p190B in the mammary gland induces hyperplastic lesions during pregnancy. Hence, we hypothesized that p190B may contribute to mammary gland tumorigenesis and metastasis. Homozygous deletion of p190-B results in central nervous system defects leading to perinatal lethality. Therefore, p190B heterozygous mice were crossed into an MMTV-Neu breast cancer model to study how loss of p190B affects breast cancer development. Preliminary data suggests that p190B heterozygosity delays the onset of primary tumor formation. However, this is not due to a change in tumor growth. Interestingly, p190B heterozygous mice tend to have a decreased number of palpable tumors and a trend toward increased formation of the primary preneoplasias. These data suggest that p190B may affect progression of preneoplastic lesions into palpable tumors. p190B heterozygous mice show a trend toward a reduced percentage of mice with lung metastasis and a reduced number of lung metastases per mouse not including mice with no metastases. However, more data is required to determine whether this trend will prove to be statistically significant. Lastly, to determine if loss of p190B induces inherent changes in signaling, we also looked to elucidate a p190B signaling pathway in the MMTV-Neu tumors. Through analysis of western blots of proteins downstream of p190B, we found that as compared to total protein loading control, ERK, loss p190B in MMTV-Neu tumors causes decreased levels of pROK, an important downstream effector of this pathway. There were also changes in individual tumors levels of ROK, pAKT, AKT, FAK, and the ratio of pROK/ROK, pAKT/AKT, and pERK/ERK. Thus, loss of one copy of p190B is sufficient to affect downstream Rho-signaling in MMTV-Neu tumors.

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P35-11: CHARACTERIZATION OF THE ROLE OF FKBP52 IN CANCER CELL GROWTH AND METASTASIS

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Cancer metastasis, rather than the growth of the primary tumor is the major reason for treatment failure, morbidity, and death among cancer patients. To identify novel molecular modulators of breast cancer metastasis, we utilized highly bone metastatic clones and sublines of the human breast cancer cell line, MDA-MB-231, in experimental metastasis models. FKBP52, a component of the Hsp90 molecular chaperone complex that is involved in the binding of unliganded steroid receptors, was identified as being expressed at higher levels in the highly bone metastatic clones and sublines through the use of gene array technology. Therefore, the objectives of this study were to determine the role of FKBP52 in cancer cell growth and metastasis. To achieve this cell line, models were generated with different expression levels of FKBP52. Antisense and sense constructs of FKBP52, as well as a non-Hsp90 binding mutant of FKBP52 (FKBP52-K354A) were stably transfected into cell lines. An antisense FKBP52 construct was stably transfected into the highly bone metastatic cell subline, MDA-MB-231SA, producing a 50% reduction in FKBP52 expression in this cell type.

In addition, overexpression constructs (wild-type and non-Hsp90 binding mutant) were transfected into the MDA-MB-231 cells and the MDA-MB-435 cell line (recently identified as the M14 melanoma cell line) where overexpression of FKBP52 produced a 2–3-fold increase in the protein level. Results utilizing the antisense cell line, MDA-MB-231SA FKAs, have demonstrated that reduced FKBP52 expression results in decreased anchorage-dependent growth under conditions of stress, as well as a decreased ability of the MDA-MB-231SA FKAs cells to form colonies in colony forming assays and in soft agar, an anchorage-independent assay. Analysis of the mechanism of action within these cells indicates that this reduction in growth is not a result of increased apoptosis, analyzed through DAPI staining and flow cytometry, but rather an alteration in the ability of the cells to progress through the cell cycle. Preliminary data indicates that MDA-MB-231SA FKAs cells have a marked alteration in tubulin localization as compared to MDA-MB-231SA Puro CTL (vector control) cells. To support this finding, mouse embryonic fibroblasts (MEFs) from FKBP52 knockout mice were obtained and showed a reduction in anchorage-dependent growth and colony forming abilities. Interestingly loss of FKBP52 in the MEFs sensitizes them to the effects of microtubule disrupting agents, such as paclitaxel. Overexpression of FKBP52 has identified a role for FKBP52 in EGF mediated migration of the MDA-MB-231 cells, as well as indicating that the interaction of FKBP52 with Hsp90 is important in the anchorage-independent growth of these cells. In the MDA-MB-435 cells, overexpression of the non-Hsp90 binding mutant of FKBP52 results in an increase in the ability of these cells to form colonies in colony forming assays. These results suggest that the role of FKBP52 is multifaceted and may be dependent on the cell model utilized. These studies are the first to demonstrate a direct role of FKBP52 in cancer cell growth and metastasis and as such identify FKBP52 as a potential novel target for cancer therapy.

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P35-12: ROLE OF PROSTAGLANDIN E2 RECEPTORS EP1 AND EP2 IN HUMAN MCF-7 Cox-2 CLONE 10 BREAST TUMOR CELL PROLIFERATION AND AROMATASE EXPRESSION

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To evaluate the role of cyclooxygenase-2 (Cox-2) in breast tumorigenesis, we developed a model of highly invasive and angiogenic estrogen-dependent human breast tumor cells. This unique cell clone, which has been designated as MCF-7/Cox-2 Clone 10, was developed by isolating stable clones from MCF-7 human breast cancer cells that had been transfected with human Cox-2 cDNA. Cox-2 is the enzyme responsible for production of prostaglandin E2 (PGE2), which has been associated with poor prognosis and invasive potential of human breast tumors. Since PGE2 binds to one of four prostanoid receptors EP1, EP2, EP3, and EP4, the present studies focused on evaluating the levels of mRNA and protein expression of the four known prostanoid receptors and determining the effects of agonists and antagonists of EP1 and EP2 on biological activities of MCF-7/Cox-2 Clone 10 human breast tumor cells including their proliferation and their role in regulation of the enzyme aromatase, which biotransforms androgenic hormones to estrogen and estradiol. The MCF-7/Cox-2 Clone 10 cell line expressed elevated mRNA levels of EP1 and EP2 (p<0.01) in comparison to the MCF-7/vector control. In contrast, EP3 mRNA was not detectable, and although both MCF-7/vector control and MCF-7/Cox-2 Clone 10 breast tumor cells expressed EP4 mRNA, the presence of Cox-2 cDNA did not alter the gene expression of EP4. In contrast, western blotting studies revealed that the MCF-7/Cox-2 Clone 10 human breast tumor cells had significantly elevated EP1 protein (p<0.05, 1.3-fold increase) compared to MCF-7/vector control cells, significantly higher levels of EP2 protein compared to MCF-7/vector control cells (p<0.01, 3.1-fold increase), significantly elevated levels of EP3 protein (p<0.05, 67-fold increase), and significantly lower levels of EP4 protein (p<0.01, 4-fold decrease) compared to MCF-7/vector control cells. Exposure of MCF-7/Cox-2 Clone 10 breast tumor cells to the agonist of EP1 (17-phenyl trinor PGE2) as well as the agonist for EP2 (Butaprost) resulted in increased cell proliferation, as well as stimulated gene expression of the P450 Cyp19 enzyme aromatase, with selective increases in mRNA of the promoter I.7 region of the aromatase gene. Both the EP1 antagonist, SC-19220, and the EP1/EP2 mixed antagonist, AH-6809, significantly decreased proliferation in the MCF-7/vector control and the MCF-7/Cox-2 Clone 10, which was consistent with both cell lines having detectable EP1 and EP2 receptors. In addition, the EP1/EP2 antagonist AH-6809 decreased gene expression of the P450 Cyp 19 aromatase enzyme specifically in the promoter I.7 region in both the MCF-7/vector control and the MCF-7/Cox-2 Clone 10. The present studies suggest a role for both EP1 and EP2 as important regulators of breast cancer cell proliferation. Additionally, signaling mediated through activation of EP1 or EP2 may regulate aromatase expression, specifically through the use of the promoter I.7 region of the aromatase gene.

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P35-13: MACROPHAGE HETEROGENEITY AND REVERSAL OF TUMOR EDUCATION

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Tumor-educated macrophages play a central role in facilitating tumor growth and metastasis. Our recent data suggest that behaviors elicited by tumor education arise from discrete populations of tumor-educated macrophages and can be reversed. When we treated mice bearing mammary tumors with intratumor GM-CSF, we observed a phenotype switch in the tumor-educated macrophages—a switch from pro-angiogenic behaviors to ones that inhibited vessel and tumor growth. This change is marked by the expression of the soluble VEGF receptor and results in slower tumor growth, reduced metastasis, lower tumor oxygen levels, and fewer, more normalized blood vessels within the tumor. Contrary to what one might predict, elimination of macrophages from similar tumor-bearing mice using a genetically engineered macrophage ablation model accelerated tumor growth and metastasis. These results may stem from the biological effects of macrophage ablation, which include a reactive increase in monocyte levels. Our data suggest that certain groups of infiltrating monocytes may perform more detrimental roles in tumor progression than other monocyte-macrophage populations, which might act in ways that benefit the host.

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P35-14: THE ROLE OF Src IN MAMMARY EPITHELIAL TUMORIGENESIS

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The levels and activity of Src non-receptor tyrosine kinases are frequently found to be aberrantly elevated in mammary carcinoma cells compared to nontumorigenic mammary epithelial cells. However, the role that Src family kinases play in tumor progression remains unclear. The overall objective of this study is to better elucidate the role that endogenous c-Src plays in mammary epithelial tumorigenesis. Our lab has found that genetic and pharmacological inhibition of Src in tumor cells that mimic the early stages of tumorigenesis (T4-2 cells), when cultured in a protein-rich extracellular matrix (3D-rBM; MatrigelTM), formed organized, polar, multicellular spheroid structures (termed “acini”) similar to the physiological lobular-aveoli structures found in the mammary tissue. Additionally, more invasive carcinoma cells (MDA-MB-231 cells) whereby Src signaling was pharmacologically or genetically inhibited were unable to form actin-rich invasive structures in 3D-rBM culture.

To further characterize the effects that Src inhibition has on tumor cell invasion, we isolated purified pools of MDA-MB-231 expressing EGFP alone or an EGFP-tagged dominant-negative mutant version of Src (EGFP-dnSrc; K295R, Y527) by flow cytometric cell sorting. Using transwell migration and invasion chamber assays, we found that MDA-MB-231 expressing EGFP-dnSrc displayed decreased migratory and invasive capabilities. EGFP-dnSrc expressing MDA-MB-231 cells also displayed a slower rate of attachment to various cell culture substrates such as plastic, laminin, and MatrigelTM. Somewhat surprisingly, we did not detect significant differences in proliferation rates between cells expressing EGFP alone and cells expressing EGFP-dnSrc, indicating that Src plays a greater role in migration and invasion than proliferation in MDA-MB-231 cells. Preliminary analysis of members of the Rho family of GTPases also showed that levels of Rho[GTP] but not Rac[GTP] or CDC42[GTP] were decreased in MDA-MB-231 cells expressing EGFP-dnSrc when incubated in 3D-rBM cultures.

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P35-15: A NOVEL PLATFORM FOR STUDYING SHAPE CHANGING ABILITY IN CANCER CELLS BASED ON MICROPATTERNED SUBSTRATES

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Metastasis, the migration and invasion of cancerous cells from a primary tumor into secondary tissues and organs, is the most frequent cause of death for breast cancer patients. This process requires the invading cell to squeeze through a relatively small

space between endothelial cells, which are connected laterally. The metastasizing cell initiates a complex series of biochemical events that induce the endothelial cells to retract from each other, opening a space that is, at first, large enough only for membrane blebs or pseudopods less than 1 μm in diameter to enter. The notion that the ability of cells to deform is important for metastasis is further illustrated by direct studies of the mechanical properties of cancer cells and is implicit in experimental approaches such as the Boyden chamber. In the Boyden chamber assay, the migration through small well-defined pores is used to evaluate the invasiveness of a cell. The diameter of the pores is a critical parameter in these assays thus suggesting an important role for the ability of a cell to change shape. However, there are no assays designed specifically to test shape-changing ability. We are developing an assay for shape-changing ability based on micropatterned cell culture substrates where the distribution of a cell adhesive protein such as fibronectin is carefully controlled. Most adherent cell types, when confronted with such a pattern, change their shape in a way that is defined by the protein pattern within limits of their ability to change shape. Thus, the approach is to challenge the cells with various limiting shapes to determine their ability to adopt. In this initial study, lines of fibronectin of decreasing width were used, effectively confining cells in one dimension. As initial model systems for developing this assay, we have examined the shape-changing ability of Swiss 3T3, B16-F1, and B16-F10 cells. As a metric for shape change, we measured the width or the length of a cell as a function of line width. The cells varied particularly in the extent to which they spread along the length of the lines. On lines $\leq 20 \mu\text{m}$ wide, 3T3 cells extended to 3–4 times their unconfined length and in some cases approached 300 μm in length. In contrast, there was no difference in length of the B16 cells at the different line widths. Further, there was no change in the heights of either B16 cell type, and thus, these cells reduce their volume approximately 10-fold on the narrowest lines. Thus, these studies reveal a dramatic difference in shape-changing ability between cancerous and noncancerous cells and suggest that the proposed assay can be used to study factors that influence shape-changing ability. Further, the results suggest interesting mechanisms for cellular responses to spatial distributions of extracellular matrix proteins. This assay for shape-changing ability has broad application to breast cancer, and we anticipate incorporating it into our related breast cancer studies.

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P35-16: BREAST TUMOR KINASE-MEDIATED BIOLOGY IN BREAST CANCER TUMORIGENESIS AND PROGRESSION

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A major method of classifying breast tumors depends on the expression of steroid hormone receptors, resulting in the development of anti-steroid hormone treatment regimens. Initially, this treatment scheme is effective. However, tumors that emerge during regression are often hormone refractory. Several protein families have been implicated in the progression of hormone refractory tumors, one of which is the protein tyrosine kinases (PTKs). PTKs regulate diverse cellular functions such as proliferation, differentiation, and apoptosis, and for this reason, they have become targets for new therapeutic strategies. Breast tumor kinase (Brk) is a novel non-receptor tyrosine kinase originally cloned from a metastatic breast tumor and is frequently endogenously overexpressed in breast cancer cell lines and approximately 85% of human breast tumors. Brk and the murine ortholog Src-like intestinal kinase (Sik) are expressed in rapidly turning-over cells in the human and mouse, respectively, and are overexpressed in colon carcinomas and cell lines. PTK6, an independently isolated, identical cDNA, is also expressed in human keratinocytes. The mechanism of Brk/PTK6-mediated tumorigenesis or progression remains unclear.

In this study, stably expressed Brk/PTK6 shRNA is used to elucidate heregulin-beta 1 induced, Brk/PTK6-mediated signaling and phenotypes in T47D breast cancer cells. Decreased phosphorylation of p38 MAPK and Erk5 was seen in Brk/PTK6 knock-down cells when compared to cells expressing non-specific shRNA, as well as a decrease in migration and proliferation. In addition, mammary gland biology in the mouse is investigated by inducible, tissue specific overexpression of a Brk/ptk6 transgene driven by the whey acidic protein (WAP) promoter. In our mouse model, initial results suggest that upon forced weaning of pups, Brk/PTK6 transgenic mice experience delayed involution. Mammary tumorigenesis in Brk/PTK6 mice also occurs but at a low incidence and in mice ages 12–13 mos, suggesting the need for a cooperating factor(s). Overall, Brk/PTK6 appears to be promoting malignant phenotypes by regulating proliferation or survival of mammary epithelial cells, both in vitro and in vivo, and may play a significant role in breast cancer tumorigenesis and/or progression.

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CELL MIGRATION/ INVASION I

Poster Session P36

P36-1: THE ROLE OF ADAM-9 IN BREAST CANCER CELL MIGRATION

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Tumor cell migration is a complex event mediated by the genetic characteristics of the tumor cells themselves, as well as the signaling molecules on the surface of, and secreted by, adjacent-activated stromal cells participating in inflammatory and wound-healing responses. In studies of the activated stromal cells of the liver, our lab discovered a secreted isoform of ADAM (A Disintegrin And Metalloprotease) 9, which, when used to stimulate MDA-MB-231 breast cancer cells, enhanced their invasive capacity through a simulated basement membrane. ADAM9 has been shown to be upregulated in 100% of breast cancer tumors evaluated in a 2005 study and the chromosomal region (8p10-11) on which ADAM9 (-Long and -Short) is located is amplified in a number of breast cancers and cell lines. These observational data, as well as work in the prostate and skin cancer fields implicates ADAM9 in cancer initiation and progression, but little is known about the function of the protein on a molecular level and the effects of ADAM9 signaling on cell migration. ADAM9 is member of a family of transmembrane integrin-binding zinc-metalloproteases implicated in cell-cell and cell-matrix interactions as well as ectodomain shedding of receptors and ligands, influencing the intracellular and extracellular signaling of the cell. Using breast cancer cell lines as our model system, we are dissecting the role of ADAM9 in cell migration and evaluating which functions of ADAM9 are required for mediating migratory signaling, with the ultimate goal of identifying the signaling pathways affected by expression of ADAM9 both on the breast cancer tumor cell and by the surrounding tissue.

Using ADAM9-specific silencing-RNA constructs, we have silenced expression of ADAM9 in BT549 breast cancer cell lines. After selection, these cells are assayed for migratory capacity by counting the numbers that migrate through the porous membrane separating the upper and lower chambers of a transwell plate in response to stimulation by fibroblast-conditioned media. This silencing of ADAM9 results in an increase in BT549 migration, which is reduced by reintroduction of ADAM9-L protein into these cells using a non-silenceable mutant. By reintroducing functional mutants into silenced cells, we determine which known functions of ADAM9 are required for the observed decrease in cell migration. Reconstituting expression with a protease-deficient ADAM9 is equivalent to wildtype ADAM9 results, indicating that the protease function is not required for ADAM9 to suppress cell migration. We are currently using integrin-binding region and other functional mutants to dissect the potential signaling pathways involved in breast cancer cell migration. Surprisingly, silencing ADAM9 in a different migratory breast cancer cell line, ZR75-1, resulted in a decrease in migration. This result leads to the interesting hypothesis that the genetic context in which ADAM9 operates may determine what effect ADAM9 has on breast cancer cell migration. We are currently using the same assays described for BT549, and in multiple other breast cancer cell lines to isolate this effect and determine the cause of these opposing phenotypes. ADAM9 has recently been identified as a potential target of drug therapy, so determining its function and the context in which it operates is an important foundation for future treatment options.

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P36-2: NOVEL ENDOPLASMIC RETICULUM PROTEIN INVOLVED IN CANCER INVASION

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Cell motility and invasiveness are critical determinants of cancer metastasis. To further illuminate these aspects of cancer dissemination, we have employed a PCR subtraction hybridization approach to identify differentially expressed genes in aggressive human HT1080 fibrosarcoma cells stimulated with concanavalin A (Con A). We identified a novel gene in ConA-stimulated as compared to control cells that is intimately involved in cancer cell migration and invasion, hence named Cancer-Related Invasion Gene (CRIG). This gene is identical to a KIAA gene reported in the HUGE database and encodes a 150 kDa protein. However, CRIG gene function, particularly in terms of cancer cell migration/invasion, has not been previously reported. Using tissue RNA arrays and mining DNA microarray databases, we observed that expression of the CRIG gene is normally limited to the central nervous system and lymph nodes. Upregulation of the obscure CRIG gene in human breast and prostate cancer tissues was found to correlate with cancer grade, stage, and recurrence. By examining human cancer cell lines, the CRIG gene is highly expressed in various disseminated carcinoma cell lines compared to less aggressive counterparts. Overexpression of the CRIG gene in nonmalignant COS-1 cells or weakly aggressive human MCF-7 breast cancer cells significantly enhanced cell migration and invasion. Downregulation of the CRIG gene in human MDA-MB-435 cancer cells using a short hairpin RNA (shRNA) approach resulted in inhibition of cancer cell migration and invasion, as examined by a Transwell Chamber migration assay and 3D type I collagen gel invasion assay. In addition, knockdown of the CRIG gene in MDA-MB-435 cells induced

a morphologic transition from fibroblast-like to epithelial-like cells, suggesting that the CRIG gene may play an important role in epithelial-to-mesenchymal transition (EMT). We further demonstrated that downregulation of the CRIG gene in MDA-MB-435 cells resulted in delayed tumor appearance and progression and prolonged survival in immunodeficient mice bearing the CRIG gene silenced-MDA-MB-435 cells as compared to luciferase shRNA MDA-MB-435 cells (control). Using rabbit polyclonal antibodies generated against the CRIG protein, we demonstrated that the distribution of CRIG is limited to the endoplasmic reticulum (ER), suggesting that CRIG may function as a chaperone of critical molecules involved in cell invasion. In conclusion, we have demonstrated that CRIG, an ER resident protein highly expressed in malignant but not nonmalignant epithelial cells, plays an important role in cancer dissemination. We propose that CRIG is a novel therapeutic target in cancer cells.

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P36-3: TIMP-1 INDUCES AN EPITHELIAL MESENCHYMAL TRANSITION VIA UPREGULATION OF THE TRANSCRIPTION FACTOR TWIST IN HUMAN BREAST EPITHELIAL CELLS

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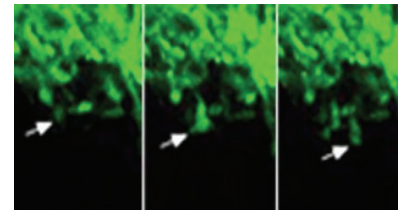
Turnover of the extracellular matrix (ECM) is regulated by the balance between matrix metalloproteinases (MMPs) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs1-4). Previous in vitro and mouse studies demonstrated that TIMP-1 reduces tumor cell invasion through MMP inhibition. Therefore, it was unexpected to discover in clinical studies that TIMP-1 overexpression correlates with a poor prognosis in certain malignancies, including metastatic breast cancer. Importantly, we and others found that TIMP-1 is a potent inhibitor of apoptosis in a variety of cell types through either an MMP-dependent or -independent mechanism. Recently, we identified CD63, a member of the tetraspanin family of proteins, as a cell surface binding partner for TIMP-1, which modulates the integrin-mediated survival pathway in the human breast epithelial cell line MCF10A. In addition, we have made a novel finding that TIMP-1 expression in MCF10A cells induces phenotypic changes in cell morphology, cell-cell adhesion, and cytoskeletal remodeling, taking on the hallmarks of an epithelial-mesenchymal transition (EMT). This is evidenced by loss of the epithelial cell adhesion molecules E-cadherin and β -catenin and an increase in the mesenchymal markers vimentin, N-cadherin, and fibronectin. Treatment with rTIMP-1 also led to a loss of E-cadherin in MCF10A cells. Interestingly, TIMP-1 signaling induced Twist expression, a transcription factor known to suppress E-cadherin gene transcription and upregulate mesenchymal markers. siRNA-mediated Twist knockdown restored E-cadherin expression in TIMP-1-overexpressing MCF10A cells, demonstrating a functional significance of Twist in TIMP-1-mediated EMT. We further demonstrated that TIMP-1 induces Twist expression and EMT in a CD63-dependent manner. Furthermore, analysis of TIMP-1 structural mutants unveiled that TIMP-1 interaction with CD63, activation of cell survival signaling, and induction of EMT in MCF10A cells does not require the MMP-inhibitory domain of TIMP-1. Taken together, we hypothesize that TIMP-1 binding to CD63 activates intracellular signal transduction pathways and leads to inhibition of apoptosis and induction of EMT in breast epithelial cells, facilitating breast cancer progression. Our study of the pleiotropic activities of TIMP-1 at the molecular level may enhance our understanding of TIMP-1's multifunctions during tumor progression and metastasis. This information may also be useful in designing more rational, mechanism-based therapeutic interventions aimed at modulating activities of MMPs and TIMPs.

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P36-4: ErbB1 AND ErbB2 HAVE DISTINCT FUNCTIONS IN TUMOR CELL INVASION AND INTRAVASATION

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The EGF receptor (ErbB1) and related family member HER2/neu (ErbB2) are often overexpressed in aggressive breast cancers and their overexpression is correlated with poor prognosis. In addition to their well-characterized contributions to cell proliferation and survival, ErbB1 and ErbB2 also contribute to other characteristics of aggressive tumors such as local invasion and intravasation, potentially independent of their effects on growth. Important for the optimization of anti-ErbB treatments in cancer is a clear in vivo identification



of the specific tumor properties that are dependent on ErbB1 and ErbB2. We differentiate the contributions of ErbB1 and ErbB2 to the key metastatic properties of in vivo tumor cell invasion and intravasation. We report that ErbB1 inhibition rapidly inhibits tumor cell motility and invasion but not intravasation while ErbB2 inhibition rapidly blocks intravasation. These experiments enable us to temporally and molecularly separate two key stages in tumor cell entry into blood vessels: invasion and intravasation. We also introduce a technique for high-resolution, long-term imaging of the tumor microenvironment, which would allow us to delineate interactions of migrating tumor cells with stromal components to study in vivo tumor cell migration and intravasation.

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P36-5: CADHERIN-11 AND ITS ROLE IN BREAST CANCER PROGRESSION AND INVASION

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Breast cancer is predicted to kill more than 44,000 women this year in the United States alone. Breast carcinomas express many factors that may contribute to their ability to proliferate or invade certain tissues, including cellular adhesion factors. Cellular adhesion has been shown to influence cell proliferation, differentiation, motility, structure, and apoptosis. One such molecule is known as cadherin-11. Cadherin-11, or osteoblast/OB-cadherin, is a type II classical cadherin that exists in both an intact and a variant isoform, which differ only by their intracellular domains. The wild-type isoform is membrane bound and provides strong homotypic cell-cell adhesion, whereas the variant is thought to be secreted into the surrounding extracellular matrix (ECM). Secreted cadherin-11 variant protein, residing in the ECM, may promote a type of cell-matrix interaction by providing an alternate binding site for cells expressing wild-type cadherin-11. In this manner, cadherin-11 may advance tumor invasion by allowing breast cancer cells to navigate through the surrounding matrix.

Preliminary data in the lab indicates that cadherin-11 may be a potential therapeutic target in breast cancer. Lab results reveal abundant cadherin-11 expression in aggressive breast cancer cell lines, as well as tissue samples derived from both advanced breast carcinomas and bone metastases. However, cadherin-11 is not expressed in normal breast epithelium or nonmetastatic breast cancer cell lines. Our lab has shown that overexpressing exogenous cadherin-11 enhances proliferation of and induces invasive capability in nonmetastatic, cadherin-null SKBR-3 breast cancer cells. Conversely, our studies demonstrate that endogenous cadherin-11 mediates proliferation in aggressive MDA-MB-231 breast cancer cells, anchorage-independent growth in soft agar, migration as well as their invasion through matrigel matrix in vitro. In addition, reduced expression of endogenous cadherin-11 in these metastatic breast cancer cells prevents subcutaneous tumor formation in nude mice. Taken together, the collective data representing both overexpression and removal of cadherin-11 in various breast cancer cell lines, respectively, confirms that the cadherin-11 adhesion molecule promotes malignant invasion and potentially tumorigenic capability of breast cancer cells. Further investigation of both cadherin-11 isoforms, particularly through the use of metastatic animal models, will be completed in an attempt to initiate progress toward a viable treatment for this terminal disease.

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P36-6: DIFFERENTIAL INVASIVE AND PROLIFERATIVE CD44 PHENOTYPES DEPENDENT ON HYALURONAN PRESENTATION

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Metastatic progression to secondary sites depend on the ability of tumor cells to migrate across the extracellular matrix to access the vasculature or lymphatics. Here, we present evidence that the extracellular matrix suar hyaluronan alters invasive phenotypes and cellular proliferation depending upon the manner in which is presented to the cell. Collagen-embedded hyaluronan inhibits filopodia formation, cellular spreading, and invasion while promoting proliferation. Alternatively, hyaluronan solubilized in cellular media promotes filopodia formation and invasion while inhibiting proliferation. These opposing effects are both mediated by the same adhesion receptor on the epithelial cells, CD44, which responds to hyaluronan differentially. These CD44 dependent effects are generated via differential signaling pathways initiated by embedded or soluble hyaluronan. While embedded hyaluronan effects are p42/44 ERK dependent, soluble hyaluronan effects are dependent upon activation of PAK. These results demonstrate that hyaluronan can have dramatically different effects on cellular behavior depending on its presentation in the cellular microenvironment.

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P36-7: BCAR3 INFLUENCES BREAST CANCER CELL MOTILITY BY REGULATING Cas LOCALIZATION AND DOWNSTREAM SIGNALING

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Antiestrogens such as tamoxifen are widely used in the clinic to treat estrogen receptor-positive breast tumors. Resistance to tamoxifen occurs either de novo or develops over time in a large proportion of these tumors. Antiestrogen resistance is also linked to enhanced motility and invasiveness in vitro, perhaps contributing to the increased aggressiveness and poor prognosis associated with resistant tumors. The long-term goal of our work is to develop a greater understanding of the molecular pathways that regulate both antiestrogen resistance and metastasis. In this study, we have focused on a molecule, breast cancer antiestrogen resistance-3 (BCAR3), whose overexpression is implicated in antiestrogen resistance. We have found that BCAR3 is highly expressed in multiple breast cancer cell lines where it associates with a second protein that also plays a role in antiestrogen resistance, p130^{Cas} (Cas; also known as breast cancer antiestrogen resistance-1; BCAR1). Both expression of BCAR3 and its interactions with Cas are elevated in more aggressive breast cancer cell lines. Depletion of BCAR3 expression from invasive breast cancer cell lines results in a loss of Cas at the cell membrane coincident with a decrease in cell migration and invasion. These findings suggest that interactions between these proteins at the cell periphery are important for their ability to regulate cellular processes associated with motility. The loss of BCAR3 also coincides with diminished membrane ruffling and stress fiber turnover responses following epidermal growth factor (EGF) stimulation. Together, these data indicate that BCAR3 regulates breast tumor cell motility at least in part by targeting Cas to the cell membrane, which in turn triggers changes in the actin cytoskeleton necessary for cell motility. We are currently using both gain-of-function and loss-of-function approaches to further investigate the relationship between BCAR3 expression and Cas signaling in breast cancer cell lines in vitro and in animal models of tumor growth. Understanding how the spatial and temporal regulation of BCAR3/Cas interactions controls breast cancer cell motility will potentially contribute to the development of new therapeutic strategies that target the subset of aggressive antiestrogen-resistant breast cancers that overexpress these molecules.

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P36-8: THE TRANSFORMING GROWTH FACTOR- β TYPE III RECEPTOR AND BONE MORPHOGENETIC PROTEINS: A RECEPTOR-LIGAND PAIR AND ITS IMPLICATIONS ON INVASION AND METASTASIS

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The Bone Morphogenetic Proteins (BMP) subfamily is the largest of the Transforming Growth Factor- β (TGF- β) superfamily. A role for BMP members in mammary carcinogenesis remains unclear. BMP-2 is secreted by and inhibits the proliferation of several breast cancer cell lines while an increase in the BMP type I receptor, BMPRI, is associated with high grade and poor prognosis in estrogen-positive carcinomas. To better understand BMP signaling in breast cancer cells, identification and characterization of cell surface receptors involved in relaying BMP signals are important.

The TGF- β type III receptor (T β RIII) is the most abundant cell surface receptor for TGF- β . T β RIII is ubiquitously expressed and has an emerging role during carcinogenesis. T β RIII binds all isoforms of TGF- β and superfamily member inhibin. Based on structural homology within the TGF- β superfamily and the ability of endoglin, an endothelial cell-specific homolog, to bind BMP family members, we sought to determine whether T β RIII functions as a mediator of BMP signaling.

Using radiolabeled ligands, we identified T β RIII as a cell surface receptor for multiple BMP family members, including BMP-2, BMP-4, BMP-7, and GDF-5. BMP binding to T β RIII mapped to sites overlapping with those characterized for TGF- β and inhibin. In addition, we demonstrate that T β RIII binds BMP-2 on the same order of magnitude as TGF- β 1. T β RIII increases affinity of the type I BMP signaling receptors (BMPRI-A and BMPRI-B) for BMP lowering the concentration of BMP necessary for efficient signaling. We also show that T β RIII dramatically increases signaling downstream of BMPRI-B by altering its subcellular localization.

Here we demonstrate that loss of T β RIII at both the mRNA and protein levels is a frequent and early event during breast cancer progression. In the 4T1 mouse model of breast cancer, restoring expression of T β RIII did not affect primary tumor growth but abrogated metastasis to the lungs. An important contributor to metastasis is epithelial to mesenchymal transition (EMT) converting non-motile polarized epithelial cells into motile and invasive cells. To understand the contribution of BMP and its receptor T β RIII in invasion, we treated a pancreatic cancer cell model of EMT with multiple

BMPs and assessed their ability to induce EMT and increase invasiveness. EMT was associated with loss of T β RIII expression and restoration of T β RIII expression inhibited BMP-mediated invasion. Overall, these data suggest that the inhibition of metastasis observed in the 4T1 breast cancer model may be due to T β RIII-mediated inhibition of BMP-induced invasion.

In conclusion, we have demonstrated that T β RIII is an important mediator of BMP signaling. Data presented here suggest that loss of T β RIII during mammary carcinogenesis facilitates BMP-induced invasion during late stages of mammary carcinogenesis. We are currently investigating the role of BMP in mammary carcinogenic EMT and associated metastasis. Understanding the specific contribution of both the ligand and the receptor to breast cancer progression will ultimately aid in the development of effective therapeutic strategies.

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P36-9: ALTERED INTEGRIN EXPRESSION DURING BREAST CARCINOMA EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT) IN THE PMC42 SYSTEM

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During metastatic progression, a small population of cancer cells leave the primary tumor and disseminate to secondary organs. The gain of migratory and invasive properties for these cells has been associated with loss of epithelial morphology and acquisition of mesenchymal characteristics—a process known as epithelial mesenchymal transition (EMT). Our laboratory has described features of the human breast cell line PMC42-LA that emulate stable intermediates of an EGF-inducible EMT. PMC42-LA is a stable epithelial subline of the parental PMC42 cell line (PMC42-ET) that exhibits 100% vimentin positivity. Both PMC42-ET and PMC42-LA exhibit increased mesenchymal gene expression and reduced epithelial gene expression after treatment with EGF, and thus the PMC42 system comprises a model system for EMT. Gene expression analysis of the PMC42 system using nylon filter and glass slide arrays identified α 3 integrin to be upregulated in accordance with the EMT. Given that altered integrin expression is known to be responsible for a number of aberrant cellular activities during tumor onset, progression, and metastatic dissemination, we further analyzed integrin dynamics of the PMC42 system during EMT. We monitored these integrin changes in the context of EMT with Q-RT-PCR and western analysis for epithelial and mesenchymal markers E-cadherin and vimentin, respectively. Using Q-RT-PCR we have observed increased expression of α 2 (4.7- to 5.5-fold), α 3 (3-fold), α 6 (4.4- to 4.5-fold), β 1 (2.2-fold), β 3 (2- to 5-fold) and β 4 (2- to 3.3-fold) integrins within 3-6 hr following EGF stimulation. Our next goal is to use siRNA to knock down specific integrin family members to determine if they impact migration and late-stage mesenchymal gene expression and thus have a functional role in EMT progression.

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P36-10: 14-3-3 σ REGULATES BREAST CANCER CELL MIGRATION AND INVASION

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Elucidation of global molecular events governing breast cancer progression is paramount for the development of novel, patient-tailored therapeutics. To study events important in malignant progression, we developed a breast epithelial cell culture model (HMT-3522) that recapitulates many features of basal-like neoplastic progression. The non-malignant (S1) cells form polarized acini when grown in a physiologically relevant, three-dimensional microenvironment (Matrigel) while their malignant counterparts (T4-2) form disorganized and proliferative colonies. By correcting the elevated levels of signaling downstream of EGFR or β 1-integrin using appropriate inhibitors, the T4-2 cells can be induced to undergo a "phenotypic reversion" and form polarized, growth-arrested colonies similar to S1 cells. To identify proteins that are upregulated in malignant T4-2 cells relative to S1 cells, we performed 2D gel electrophoresis and mass spectrometry analysis of lysates from S1 and T4-2 cells when cultured in Matrigel. We were able to identify several proteins that are consistently altered in abundance during the malignant progression, with the protein having the most established relevance to breast cancer being 14-3-3 σ . We validated the proteomic data by western blot analysis and further demonstrated that 14-3-3 σ expression in T4-2 cells becomes comparable to S1 cells upon phenotypic reversion with the EGFR inhibitor AG1478. Interestingly, shRNA-mediated knockdown of 14-3-3 σ in T4-2 cells did not result in phenotypic reversion but rather decreased their motility and invasiveness through Matrigel-coated transwell inserts. These data suggest a novel role of 14-3-3 σ in the regulation of breast epithelial cell migration.

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P36-11: THE ROLE OF PROTEIN KINASE D (PKD) SIGNALING IN BREAST CANCER CELL MIGRATION AND INVASION

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The serine/threonine kinase protein kinase D (PKD) has recently emerged as a key coordinator of cellular functions including Golgi trafficking, cellular proliferation, and survival. Because PKD family members (PKD1, PKD2, and PKD3) share a similar cysteine-rich C1 domain as the classical and novel PKCs, it was initially classified as a member of the PKC family. However, the catalytic domain of PKD shows little homology to that of PKCs but has instead a high-sequence similarity to members of the calcium/calmodulin-dependent protein kinase (CAMK) family and is therefore currently categorized as a novel subgroup of this family. Activated phospholipase C (PLC) produces diacylglycerol (DAG), which recruits cytosolic PKD to the plasma membrane and Golgi membranes. This binding facilitates phosphorylation of PKD's activation loop by kinases of the PKC family. Known substrates of PKD include Kidlns 220 (kinase D-interacting substrate of 220 kDa), and RIN, a negative regulator of Ras, 27-kDa heat shock protein (Hsp27), and HDACs 5 and 7. Phosphorylation of another substrate, PI4KinaseIII β by PKD is required for the fission of Golgi vesicles and proper delivery of cargo to the plasma membrane. In addition to a critical role in homeostatic cell signaling, particularly at the trans-Golgi network, it is becoming increasingly clear that PKD participates in cancer progression. Mutated PKD alleles have been identified in cancer tissue screens, and it is overexpressed in a number of cancer tissues and cell lines. Studies have implicated PKD in the migration of fibroblast cells, the recycling of integrins, and the degradation of extracellular matrix in invasive breast cancer cells. However, whether PKD is required in the migration and invasion of epithelial cells and possible mechanisms by which it may control this phenotype are unknown. My initial data demonstrate that PKD is required for efficient epithelial cell migration. PKDs 1 and 2 were knocked down using siRNA in cancer cells and were then assayed in transwell migration and in scratch wound-healing assays. A series of PKD-specific chemical inhibitors were also used. Rescue experiments involving the re-introduction of both wild-type and various mutant PKD alleles in these knockdown cells reveal the requirement of regulation by upstream kinases and regulatory motifs in cell motility. Mutants include phosphomimetic and unphosphorylatable alleles of demonstrated and putative phosphorylation sites, PKDs 1 and 2 lacking critical residues in the PDZ-binding motifs, and those mutated alleles identified in cancer tissue screens. The viability of PKD-knockdown and -inhibited cells is impaired and they become apoptotic. In addition to a decrease in cell number and changes in cell morphology, caspase cleavage is evident in cell lysates by western blotting. Fluorescence activated cell sorting will be used to further investigate the effect of PKD knockdown and inhibition on cellular proliferation and viability.

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P36-12: ROLE OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 5B IN BREAST CANCER CELL MIGRATION

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Signal transducer and activator of transcription (STAT) 5b is a transcription factor involved in pro-proliferative and pro-survival signaling in a number of solid tumors, including breast cancer. While STAT5b has been shown to be essential for invasion of metastatic prostate cancer cells, its role in breast cancer metastasis has not been determined. This work aims to elucidate the role of STAT5b in breast cancer cell migration, invasion, and metastasis. We have found that STAT5b is essential for migration of BT-549 and MDA-MB-231 human breast cancer cells to serum in Boyden Chamber assays. Knockdown of STAT5b inhibits migration of these cell lines by 60%–80%. This inhibition is not due to a defect in cell viability or adhesion. Furthermore, STAT5b siRNA inhibits migration across a range of serum concentrations (0.1% to 10% serum). Knockdown of STAT5b also inhibits invasion of these cells through Matrigel-coated filters in a Boyden Chamber assay. Importantly, inhibition of migration upon STAT5b knockdown can be rescued by re-introduction of wild-type STAT5b. Interestingly, a transcriptionally inactive Y699F-STAT5b mutant can also rescue migration to the same extent as wild-type STAT5b. These results suggest that the transcriptional activity of STAT5b is not required for its promigratory function. In summary, STAT5b is important for migration and invasion of breast cancer cells and may have a novel, nontranscriptional function in promoting tumorigenesis. Further elucidating the mechanism by which STAT5b promotes migration and invasion of breast cancer cells may lead to more effective therapies for preventing and treating metastasis.

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P36-13: SUPPRESSION OF CARCINOMA CELL INVASION BY SYNDECAN-1

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Background: Syndecan-1 (Sdc1) is predominantly expressed in the epithelial cells. Via its heparan sulfate chains, it can bind to various ligands, including extracellular matrices, such as laminins and collagens. The expression of Sdc1 is highly regulated during the epithelial development and under some pathological situations such as wound healing and cancer. It has been proposed that Sdc1 might bind to actin cytoskeleton and modulate epithelial morphology. However, the role of Sdc1 in the cell polarity, one of the most characteristic features of epithelium, is still largely unknown.

Objective: The major aim of this proposal is to examine how Sdc1 regulates polarization of mammary epithelial cells during tumorigenesis. In the preliminary studies, Sdc1 causes highly invasive mammary gland carcinoma MDA-MB-231 cells cultured in three-dimensional (3D) matrigel to become cohesive and polarized. The hypothesis of this project is that Sdc1 specifically titrates the invasion signaling activated by $\alpha 3 \beta 1$ integrin leading to cell cohesion.

Methodologies: Cells were cultured in 3D matrigel or collagen to mimic the malignant growth of breast cancer in vivo.

Results: In contrast to parental cells, human MDA-MB-231 cells overexpressing Sdc1 revert from an invasive to a polarized, cohesive phenotype characterized by increased cell-cell adhesion and cortical expression of F-actin. Sdc1 itself is localized to the cell-cell junctions, along with phosphatidylinositol-3-kinase (PI-3K) and, surprisingly, E-cadherin. The MDA-MB-231 cells are normally negative for E-cadherin

due to hypermethylation of its promoter; these data suggest that Sdc1 activates a pathway that overcomes this block.

The Sdc1 cohesion activity is highly dependent on the cells being cultured in 3D and being in matrigel, implicating a role for a specific matrix component in the matrigel; cells cultured in collagen gels remain invasive. Blockade of the $\alpha 3 \beta 1$ integrin, an integrin that would recognize laminin in matrigel, partially mimics the activity of Sdc1 overexpression; conversely, enhancing the activation of $\beta 1$ integrins reverts Sdc1-overexpressing cells back to the invasive phenotype, suggesting that Sdc1 inhibits $\alpha 3 \beta 1$ integrin signaling leading to cell invasion. One means for the $\alpha 3 \beta 1$ integrin to cause cell invasion is via its activation of the GTPase Rac1. Indeed, Sdc1 overexpression suppresses the signaling pathway leading to Rac1 activation, although it does not block other signals from the $\alpha 3 \beta 1$ integrin.

The blockade of Rac1 by Sdc1 is required but not sufficient for reversion of the invasive phenotype. Sdc1 also activates and recruits PI-3K to the cell-cell junctions. This activation of PI-3K is required, as Sdc1 fails to promote the cohesive phenotype in the presence of the LY294002, a specific PI-3K inhibitor. We find that the enhanced expression of E-cadherin that occurs when Sdc1 is overexpressed is dependent both on the reduction in Rac1 activity and the activation of PI-3K.

Conclusions: This study offers a unique system to examine the downstream signaling of Sdc1, and it provides a new mechanism for Sdc1 regulation of tumorigenesis. This reveals for the first time the underlying mechanism of crosstalk between Sdc1, E-cadherin, and the $\alpha 3 \beta 1$ integrin.

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METASTASIS I

Poster Session P37

P37-1: BREAST CANCER GROWTH AND METASTASIS IN A RAT MODEL OF TYPE 1 DIABETES MELLITUS

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Background and Objectives: Findings from two large prospective epidemiological studies (with over 1 million subjects in each) show that diabetes mellitus is an independent risk factor for death from breast cancer (Coughlin et al, 2004, *Am. J. Epidemiol.* 159: 1160; Jee et al, 2005, *JAMA* 293: 194). The mechanism to explain how diabetes increases mortality from breast cancer is highly speculative. Most studies on interactions between diabetes and breast cancer have focused on type 2 diabetes which differs from type 1 diabetes in 2 ways. First, unlike type 1 diabetes, in type 2 diabetes hyperinsulinemia occurs and is associated with high levels of IGF-1, which is known to increase cancer growth. Secondly unlike type 1 diabetes in which weight loss is a problem, type 2 diabetes is associated with obesity. Our current studies focus on type 1 diabetes and breast cancer. In a preliminary study, tail vein injection of breast cancer cells (MADB106) into type 1 diabetic rats showed larger lung metastases, compared to injection into normoglycemic rats. Perhaps, breast cancer metastasis increases in the presence of diabetes and contributes to increased mortality reported in epidemiological studies. The objectives here are to determine if:

1. Type 1 diabetic rats have larger and more numerous lung metastases compared to normoglycemic rats,
2. Type 1 diabetic rats have larger tumors following injection of breast cancer cells into the mammary fat pad compared to normoglycemic rats, and
3. Type 1 diabetic rats have deficiencies in natural killer (NK) cells, responsible for killing MADB106 cells, compared to normoglycemic rats.

Description of Methodologies: To achieve the objectives, Fisher 344 rats are injected either with streptozotocin to induce type 1 diabetes or saline to serve as normoglycemic controls. To test for differences in size and number of metastases, syngeneic MADB106 rat breast cancer cells known to specifically metastasize to the lungs are injected into the tail veins of diabetic and control rats. Fourteen days later, lung metastases are measured and counted. To evaluate tumor growth independent of metastasis, the MADB106 breast cancer cells are injected into the mammary fat pads of diabetic and control rats, then tumor size is determined 14 days later. To test for differences in NK cells, both the numbers of blood NK cells and NK cell killing activity are determined in diabetic and control rats 14 days after MADB106 breast cancer cell injection into the tail vein.

Results to Date: Our findings show that rats with type 1 diabetes mellitus have larger and more numerous lung metastases following the tail vein injection of MADB106 breast cancer cells compared to normoglycemic rats ($p < 0.05$). However, there is no difference in the size of the mammary fat pad tumors. In progress studies show no differences in NK cell number or activity in diabetic rats, suggesting that deficiencies in these cells may not be responsible for the increased metastasis.

Conclusions and Impact on Breast Cancer Research: The results suggest that metastasis, but not tumor growth, are increased in type 1 diabetic rats. It remains unclear as to the mechanism(s) involved. Perhaps vascular changes are responsible for the differences in metastasis.

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P37-2: NOVEL APPROACHES TO TARGET THE COX-2 PATHWAY TO REDUCE BREAST CANCER METASTASIS

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Breast cancers frequently express high levels of COX-2, which is associated with heightened metastatic capacity and poorer overall survival. The COX-2 product PGE2 acts through a family of four G-protein coupled receptors designated EP1-EP4. These four receptors mediate intracellular signaling through multiple pathways. We have shown that murine and human malignant breast cells express EP2, EP3, and EP4 on the cell surface, and all four EP receptors are detected in the cytoplasm. We tested the hypothesis that prevention of EP receptor signaling would, like inhibition of PGE synthesis, inhibit breast tumor metastasis. We now show that pharmacologic antagonism of the EP4 receptor with either AH23848 or ONO208 is protective against metastatic disease. Likewise, gene silencing of EP4 using an shRNA to specifically target EP4 also results in reduced tumor metastasis. Natural killer (NK) cells are potent inhibitors of tumor metastasis. To determine if NK cells contribute to antime-tastatic activity of EP4 antagonists, we compared therapy in immunologically intact and NK-depleted mice. Metastasis inhibition by EP4 antagonism was lost in mice lacking functional NK cells. Consistent with these findings, EP4 antagonism enhanc-

ed the ability of NK cells to lyse tumor target cells in vitro. NK cells are activated in response to loss of major histocompatibility complex (MHC) class I expression on target cells. EP4 antagonism inhibited MHC class I expression in a dose-dependent manner in parallel with the enhanced NK-sensitivity to lysis. Thus, EP4 is a potential therapeutic target to control breast cancer metastasis, and the therapeutic response resulting from antagonism of this receptor depends on active participation by host NK cells. Taken together, these studies support the hypothesis that EP receptor antagonists may be an alternative approach to the use of COX inhibitors to prevent breast tumor progression.

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P37-3: SELF-SEEDING: A NOVEL MECHANISM LINKING TUMORIGENESIS AND METASTASIS

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The strong association between tumor size and metastatic risk has been frequently observed in breast cancer. However, the nature of this link remains unclear. In a conventional model of cancer, cancer progression is segregated into two independent processes, namely tumorigenesis and metastasis. In this model, it is thought that increased ratio of cell proliferation to cell death accounts for tumorigenesis while progressive accumulation of favorable mutations results in metastasis, not necessarily contributing to tumor growth. However, several recent reports have provided a link between these two processes. For example, using a xenograft mouse model, our group has identified a gene signature that promotes primary tumor growth and mediates metastasis as well, providing a functional link between primary tumor size and metastasis. Some of the genes in this signature have been shown to mediate in situ growth through mechanisms such as angiogenesis. On the other hand, many other genes mediate distinct functions such as intravasation/extravasation, which is more closely associated with tumorigenic colonization from the circulation rather than with direct in situ expansion of a tumor mass. This suggests that in addition to the direct effect on tumor growth, cells expressing these mediators of metastasis might contribute to the tumor growth via a new mechanism. We have recently proposed a "self-seeding" hypothesis as a new mechanism for this consistent link between primary tumor size and metastatic behavior. In this process, we hypothesize that metastatically competent cells escape and re-seed the primary tumor mass thereby promoting overall growth by using overlapping genes, if not the same, which mediate metastasis to a distant organ.

In the current study, we demonstrate the self-seeding phenomenon by using a xenograft model with a breast cancer cell line, MDA-MB-231, as well as pleural effusion samples from breast cancer patients. We find that highly metastatic cancer cells exhibit better self-seeding abilities than poorly metastatic cells, indicating that genes required for seeding of distant organs are also necessary for self-seeding. In addition, we show a seeding from metastases to a primary tumor. Importantly, we provide evidence that self-seeding contributes to the growth of primary tumor. Possible mechanisms on how self-seeding confers the growth advantage of the primary tumor are being examined. These findings demonstrate the existence and the role of self-seeding in breast cancer. Furthermore, this novel mechanism could link the previously disparate processes of tumor growth and metastasis. Finally, this study could provide a therapeutic implication of self-seeding.

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P37-4: NATURAL CARBOHYDRATE INHIBITORS OF GALECTIN-3 FOR THE PREVENTION OF BREAST CANCER METASTASIS

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At the time of diagnosis, many patients with primary breast cancer have disseminated tumors. Therefore, surgery alone is not the treatment of choice and the use of chemotherapy as an adjuvant is preferred. However, although this combination treatment is initially beneficial, most tumors progressively develop drug resistance, and eventually it fails to prevent cancer metastasis. As most cancer processes leading to metastasis include surface phenomena involving angiogenesis, homotypic aggregation, and heterotypic adhesion to the endothelium, selective inhibition of such surface molecules responsible for the abovementioned mechanisms may constitute an effective approach to prevent cancer metastasis. Galectin-3 (gal3), a member of the beta-galactoside-binding gene family, is involved in the progression and metastasis of a variety of cancers, including breast tumors. Moreover, gal3 secreted by tumor cells induces apoptosis of cancer-infiltrating T cells, thus playing a role in the immune escape mechanism during tumor progression. Recent studies demonstrate that hematogenous cancer metastasis originates from intravascular growth of endothelium-

attached rather than extravasated cancer cells, underscoring the key role of tumor-endothelial cell interactions in cancer metastasis. In this multi-step adhesion of tumor cells to the vasculature endothelium, endothelium gal3 participates in docking of cancer cells including breast cancer on capillary endothelium. For this docking process, cancer cells-associated Thomsen-Friedenreich disaccharide (TFD) plays a leading role by specifically interacting with endothelium-expressed gal3. Therefore, in cancer progression and metastasis, gal3 has become a very important player and, thus, exogenous TFD would block gal3-mediated homotypic aggregation and tumor cells-endothelial interactions preventing cancer metastasis. Further, these carbohydrates would also block secreted gal3 mediated T-cell (cancer infiltrating) apoptosis, thus facilitating antitumor immune responses. The objectives of this study are (1) to test the ability of these carbohydrate blockers (TFD containing compounds) of gal3 to inhibit tumor cell growth and adhesion to endothelium and (2) to test the ability of these TFD-containing compounds to inhibit T cell death. For this purpose, we have expressed recombinant gal3 and affinity purified. We have tested a TFD containing crude extract from a natural source for the inhibition of gal3 binding on a solid phase assay. Results indicated that this extract is about threefold better than the citrus pectin in blocking gal3 binding activity. The extract was found an effective inhibitor of gal3-mediated cancer cell adhesion to endothelial cells. Purification of TFD containing glycopeptides and their abilities to inhibit T cell apoptosis are under way.

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P37-5: NOVEL QUANTITATION OF AUTOCRINE STIMULATION OF CELL MOTILITY IN VITRO AND METASTASIS IN VIVO OF HUMAN BREAST CANCER CELL LINES

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This work explores the undescribed effects of different forms of the L1 adhesion/recognition molecule on breast cancer cell motility. Breast cancer cells metastasize to a variety of secondary sites, including brain, which is ultimately responsible for patient death. A recently discovered mechanism of proteolysis or "shedding" of the L1 adhesion molecule extracellular domain (ectodomain) results in autocrine/paracrine stimulation of motility in ovarian and uterine cancers and causes of high mortality rates. L1 is expressed and shed abnormally in some breast cancer cells along with cognate integrin receptors, so such an autocrine mechanism is now being explored. Also, breast cancer cells entering into the brain environment rich in axonal L1 may stimulate spreading in the brain, and may contribute to the 50% mortality of patients with brain metastases. This is an unexplored concept in the progression of breast cancer, and these studies are highly likely to provide new insights into the aspects of this disease that contribute to patient mortality. This novel mechanism may account for the ability of human breast cancer cells to express and shed the L1 adhesion molecule as a means to stimulate their own motility and metastasis. L1 expression in several commercially available breast cancer cell lines is being documented by western blot analyses using several antibodies to different L1 regions (i.e., ectodomain versus cytoplasmic domain). L1 expression in individual cells is being documented by immunocytochemistry and flow cytometry. Cell lines have been found that either do or do not express L1. In vitro motility analysis of cell behavior will be done using a novel automated time-lapse microscopy system and quantitation software. Data will be compared for cells in which (1) L1 expression has been attenuated by retroviral antisense, (2) the shed L1 ectodomain is sequestered by L1-blocking antibodies, (3) integrin receptors are blocked by RGD-containing L1 peptides, and (4) L1 ectodomain is overexpressed by retroviral vectors. In addition, fluorescently labeled cells will be tracked on cell monolayers that stably express cell surface (non-shed) or shed L1 to determine the effects of different forms of exogenous L1 (like on axon tracts). In vivo studies will involve a novel chick embryo brain tumor model but where drug-resistant cells are microinjected into the blood vessels of the chorioallantoic membrane (CAM). Several days later, brains will be dissociated, plated, and drug selected for resistant cancer cell colonies. This will precisely quantitate the number of breast cancer cells that localized to brain. This assay could then be used to determine metastatic potential between different cell lines, as well as with cell lines that have been experimentally treated (e.g., antisense-L1 infected). These approaches will determine if different known forms of L1 stimulate or attenuate breast cancer motility and metastasis. Correlation of results between these two complementary approaches will establish if the novel motility assay can predict metastatic or invasive potential. Therapeutic strategies can be devised to inhibit signals resulting from this mechanism that may contribute significantly to breast cancer cell spread and metastasis to brain and elsewhere.

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P37-6: THE DIALOGUE OF METASTASIS-UNCOVERING JUXTACRINE GENETIC CASCADES WITH A TOXOPLASMA GONDII ENZYME

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Background: This project seeks to establish a strategy for detecting the transcriptional activities specific to breast cancer cells or to stromal cells without disaggregating tumors. This approach will be used to test the hypothesis that lysine oxidase (LOX) increases breast cancer metastasis in part by altering gene transcription in cells adjacent to the cancer cells. Despite the importance of cancer cell-stromal interactions, current approaches are poorly suited to analyze the effect of cancer cells on their microenvironment (and vice versa) within intact tumors. This study modifies a recently described metabolic labeling technique to determine (without cell separation) how gene transcription in bone marrow stromal cells is affected by cell-cell contact with highly metastatic ([LOX]-expressing) or poorly metastatic (LOX-knockdown) breast cancer cells. Objectives of this project are: (1) Establish a system to determine gene transcription specific to HS-27A bone marrow stromal cells without the need to isolate the cells from co-cultured MDA-MB-231 breast cancer cells (or vice versa). (2) Identify transcripts in cancer cells that depend on contiguity with bone marrow stromal cells. (3) Identify transcripts in stromal cells that depend on contiguity with breast cancer cells. Determine whether MDA-MB-231-induced transcription in bone marrow cells is LOX-dependent.

Methods: A fusion protein encoding yellow fluorescent protein and the toxoplasma gondii enzyme uracil phosphoribosyl transferase (UPRT) will be generated and its UPRT activity validated. The UPRT enzyme enables eukaryotic cells to incorporate exogenous thioracil into their RNA (Cleary et al., *Nature Biotechnology*, v23, 232-7 [2005]). RNAs synthesized during a thioracil pulse can subsequently be labeled with biotin and isolated on streptavidin beads followed by microarray analysis. Red-fluorescent MDA-MB-231 cells expressing vector control or LOX knockdown siRNA will be cocultured with eYFP-UPRT-transduced HS-27A human bone marrow stromal cells. RNAs synthesized during a thioracil pulse will subsequently be labeled with biotin and isolated on streptavidin beads, followed by microarray analysis.

Results: An eYFP-UPRT fusion gene was generated and subsequent specific RNA thiolation and biotinylation were confirmed in transfected 293T cells using streptavidin blotting of fractionated RNA. Rescue of biotinylated RNA on streptavidin beads was confirmed. MDA-MB-231 and HS27A cell lines were also stably transfected with the UPRT transgene, resulting in cytoplasmic yellow fluorescence, and red-fluorescent MDA-MB-231 cell clones were separately prepared. Red and yellow fluorescence of separate cell lines helps to visualize mixing during coculture. Groundwork is now established for planned coculture experiments, LOX knockdown, and transcription analysis.

Conclusions: Preliminary results support the feasibility of measuring molecular communication between breast cancer cells and bone marrow stromal cells at the level of cell-specific pulsed transcription for the first time. This strategy could identify novel components of a cancer-stroma communication network that are amenable to targeted therapy.

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P37-7: DOES LOSS OF NEDD9 LIMIT BREAST CANCER METASTASIS?

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NEDD9 is a scaffolding protein that regulates TGF- β , Src, FAK, Aurora A, E-cadherin, and other proteins that drive the processes of cell transformation. Recent studies show that upregulation of NEDD9 characterizes metastatic spread of melanomas, lung cancers, and aggressive forms of glioma and that exogenous overexpression of NEDD9 in vivo promotes metastasis. It is currently unknown whether NEDD9 also plays a role in early stages of cancer to predispose individuals for metastatic disease but is plausible based on its interactions. Based on these data, we hypothesized that lack of NEDD9 is likely to limit tumor metastasis and potentially other early stages of the transformation process. To test this hypothesis, we proposed crossing NEDD9^{-/-} mice to MMTV-neu mice and assessing the incidence, latency, and pathological features of metastases to the lung. We also proposed to analyze primary tumors and metastases in NEDD9^{+/+}, MMTV-neu animals using immunohistochemical analysis and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to establish the frequency and degree of altered NEDD9 overexpression. This would allow us to assess whether altered NEDD9 expression is a useful biomarker for breast cancer prognosis and whether NEDD9 is a promising candidate for the development of targeted therapies. Crosses between NEDD9^{-/-} mice and MMTV-neu mice were estab-

lished during the fall of 2007. In the MMTV-neu transgenic model, expression of human neu/ErbB2/Her2 activates pathways commonly engaged in human breast cancer, causing breast cancer and metastasis in animals from 7–12 months of age. We have accrued 25 animals into each of 4 arms for evaluation: (1) NEDD9^{+/+}, MMTV-neu; (2) NEDD9^{+/+}, ⁺/₊; (3) NEDD9^{1/-}, MMTV-neu; and (4) NEDD9^{-/-}, ⁺/₊. Based on published values for tumor latency in the MMTV-neu model, we anticipate the critical period for evaluating the consequences of NEDD9 status for tumor growth will be from March–May 2008. To enrich our study, we will contrast the results to data obtained from an ongoing study in which NEDD9 ^{+/+} and ^{-/-} status is being investigated in an MMTV-PyVT breast cancer mouse model. Because MMTV-PyVT is a more aggressive tumor, these mice have a much shorter latency until tumor development, that is, in the MMTV-PyVT transgenic mouse, breast-specific expression of the polyoma middle T oncogene causes rapid-onset primary breast adenocarcinoma that metastasizes to the lung with high incidence within 3 months. We have used a similar study design as for the MMTV-neu cross with 25 animals per study arm. To date, we have found that HEF1-negative status results in a decrease in total tumor burden.

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P37-8: A ROLE FOR MYELOPEROXIDASE IN TUMOR PROGRESSION AND METASTASIS

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Clinical studies using breast tumor samples show a strong correlation between the level of interleukin-8 (IL-8) expression and tumor progression, metastasis, and the rate of mortality.

The ectopic release of IL-8 by tumor cells attracts neutrophils to the site of the tumor. During this process, neutrophils release several enzymes that enhance their migration between the site of extravasation and the tumor. One of these enzymes, myeloperoxidase (MPO), is present at high levels and is primarily a neutrophilic enzyme. MPO coupled with neutrophilic NADPH oxidase produces hypochlorous acid (HOCl), a strong oxidant. The HOCl freely diffuses through membranes and is a potent mutagen in mammalian cells. Its ability to oxidize sulfur-containing amino acids allows it to activate latent matrix metalloproteinases (MMPs) and inactivate the tissue inhibitors of metalloproteinases (TIMPs) embedded in the extracellular matrix (ECM) thus increasing the rate of remodeling the ECM. It is hypothesized that the MPO released by the tumor-associated neutrophils contributes to tumor progression and metastasis by increasing both the rate of ECM remodeling and cellular mutagenesis.

To test this hypothesis, MMTV-neu transgenic mice, which develop early spontaneous metastatic breast tumors, will be crossed with MPO-null mice. Once MMTV-neu x MPO^{-/-} mice are produced, the progression of breast tumors in these mice will be compared with that of MMTV-neu x MPO^{+/+} mice.

If, as anticipated, these experiments demonstrate that MPO contributes to tumor progression and metastasis, we will have identified an attractive target for the development of specific, high-affinity inhibitors of these processes for the treatment of breast cancer.

At this time, the pups obtained from the F1 cross of the above genotypes are less than a week old. Once they are old enough, they will be crossed to obtain F2 in which we are expecting 12 females that are of the MMTV-neu x MPO^{-/-}. These will be monitored to determine if their tumor formation and progression are attenuated compared to the MMTV-neu.

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P37-9: ROLE OF CELL FUSION IN BREAST CANCER METASTASIS

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Background and Objective: Cell fusion is a normal aspect of mammalian development involved in fertilization, formation of tissues like placenta and muscle, and stem cell-mediated tissue renewal. Like other normal physiologic processes, cell fusion may be subverted in cancer, which may allow cancer cells to acquire traits that may serve to drive cancer progression such as aneuploidy, invasiveness or stem cell-like properties of self-renewal, plasticity, and resistance to toxins. The objective of this project is to evaluate the role that cell fusion may play in breast cancer metastasis.

Methodology: We have designed a strategy to detect cell fusion events using Cre-lox-mediated recombination. We have demonstrated the utility of this strategy to detect these events *in vitro*. We are now employing this approach to detect cell fusion events in animal models of breast cancer metastasis to isolate and characterize fused cells and to determine the metastatic potential of these cells.

Results: To date, we have demonstrated our ability to initiate tumors in the mammary fat pad and to monitor tumor growth and metastasis using bioluminescence imaging. We have validated the feasibility of several technical aspects of this proposal, including the analysis of tumor sections and cells with immunofluorescence and FACS, respectively.

Impact: The successful completion of these studies will define a new mechanism underlying metastatic breast cancer and generate new approaches to diagnosis and therapy of this disease.

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P37-10: ISOLATION AND CHARACTERIZATION OF BONE MARROW-DERIVED METASTATIC ZR-75-1 CELLS IN ANIMAL MODELS OF HUMAN BREAST CANCER

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Bone metastasis is one of the major causes of morbidity and mortality in breast cancer (BC) patients. While several molecular pathways have been shown or implicated to promote bone metastasis of human BC cells, how metastatic BC cells specifically take residence in skeletal tissue remains unknown. In this study, we have investigated whether the media conditioned by metastasis-competent BC cells or the presence of a growing tumor formed by metastasis-competent BC cells can enhance bone and lung metastasis of metastasis-incompetent BC cells. Five-week-old female athymic nude mice were injected i.p. daily with 300 μ L conditioned medium (CM) from metastasis-competent human breast cancer MDA-MB-231 or MDA-MB-435-F-L cells for 5 days prior to the intracardiac injection of metastasis-incompetent human breast cancer ER-positive ZR-75-1 cells stably transfected with a GFP expression plasmid. Injection of CM every alternate day was continued for more than 3 months. Control animals were injected with the original serum-free medium. Bone and lung metastasis detected by whole mouse GFP imaging revealed that three out of eight mice treated with CM from MDA-MB-435-F-L, compared to one out of eight mice in the control group, developed metastases to lung and bone in the absence of estrogen supplementation. This suggests that the CM might have promoted the metastatic potential of ZR-75-1 cells. When ZR-75-1-GFP cells were injected intracardially into mice bearing growing tumors formed by MDA-MB-231 or MDA-MB-435-F-L cells, we also found ZR-75-1-GFP cells in bone marrow without estrogen supplementation. These metastatic ZR-75-1 cells were flushed out of the bone marrows and established as variant cell lines. Two variant lines, called B4 and B6, from two mice were further characterized for their tumorigenicity in the mouse mammary fat pad and their metastatic potential to lung and bone through intracardiac injection in the presence or absence of estrogen supplementation. Surprisingly, although both variants were isolated from bone marrow and they showed the same anchorage-dependent and -independent growth property *in vitro*, B6 was significantly more tumorigenic and bone metastatic than B4 in both with or without estrogen supplementation *in vivo*. In fact, B6 was equally tumorigenic and metastatic to lung, brain, and bone in the absence of estrogen supplementation as B4 in the presence of estrogen supplementation. Like parental ZR-75-1 cell, B4 is not tumorigenic and metastatic without estrogen supplementation. Our study suggests that bone marrow-derived metastatic breast cancer cells may not share similar malignant property. Further characterization of these cell lines is under way to reveal the potential estrogen-dependent and -independent biomarkers of breast cancer progression.

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P37-11: THE CYSTEINE CATHEPSIN INHIBITOR STEFIN A REDUCES THE DEVELOPMENT OF DISTANT METASTASES IN BREAST CANCER

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Metastatic breast cancer is a life-threatening disease with limited treatment options. Given the lack of molecular targets for tailored therapy, we analyzed changes in gene expression associated with metastatic progression in breast cancer using a clinically

relevant murine model of spontaneous metastasis to lung and bone. From microarray expression profiling of immunopurified tumor cells derived from primary tumors and matched metastases, we identified the cysteine cathepsin inhibitor Stefin A.

In primary tumors, Stefin A expression inversely correlated with metastatic potential in 4T1-derived lines and was not detected in tumor cells in culture, indicating induction only within the tumor microenvironment. Enforced expression of Stefin A in the highly metastatic 4T1.2 cell line significantly reduced spontaneous bone metastasis in vivo following orthotopic injection into the mammary gland. Consistent with the mouse data, Stefin A expression correlated with disease-free survival (absence of distant metastasis) in a cohort of 142 primary tumors from breast cancer patients. This was most significant for patients with invasive ductal carcinoma expressing Stefin A, who were less likely to develop distant metastases (log rank test $p=0.0075$).

In human lung and bone metastases, we detected irregular Stefin A staining patterns, with expression often localizing to micrometastases (<0.2 mm) in direct contact with the stroma. We propose that Stefin A, as a cysteine cathepsin inhibitor, may be a marker of increased cathepsin activity in metastases.

Measurement of cathepsin activity in mammary tumors revealed that cathepsin B activity is increased in highly metastatic primary tumors. Additionally, cathepsin B was detected co-expressed with the inhibitor in lung and bone metastases, both in the murine model and in human tissues. We conclude that Stefin A expression reduces distant bone metastasis in breast cancer and propose that this may be due to the inhibition of cysteine cathepsins such as cathepsin B. We are currently investigating the functional role of cathepsin B on metastasis to bone.

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P37-12: OPERATIVE THERAPY AND THE GROWTH OF MICROMETASTASES: CAUSE AND EFFECT?

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Background and Objectives: Breast cancer patient recurrence and mortality data suggest that there is a perturbation of the growth of metastatic deposits that may be consequent to operative intervention. This growth has been hypothesized to be the result of the removal of antiangiogenic substances elaborated by the primary tumors or the generation of proangiogenic substances and/or growth factor/cytokines that are produced to heal the operative wound. The aim of this study was to determine if the change in serum proteins as a function of time relative to the operation to remove the primary tumor could be reliably assayed.

Methodology: Tumors were extirpated 8 weeks after inoculation of human breast cancer cells into the mammary fat pad of nu/nu mice. Immediately prior to and 24 and 48 hours, and 1 week after extirpation, blood was obtained from the facial vein of the mice. Mice were sacrificed 10 weeks after operative intervention and necropsy performed to verify the presence/absence of lung metastases. State-of-the-art proteomics, two-dimensional column chromatography coupled in-line with linear ion trap tandem mass spectroscopy, was utilized for qualitative analysis of the serum proteins.

Results: 3,458 proteins were identified over the 4 time points, 278 of these with high confidence in the accuracy of the identification, 3,174 with lower confidence. Proliferation, invasion, and inflammatory proteins were seen at all time points. Acute phase reactants were seen at 24 and 48 hours after operation and angiogenesis proteins at 24 and 48 hours as well as 1 week post-op. One of the most interesting proteins identified was MST1 (macrophage stimulating 1), a secreted glyco-protein closely related to Hepatocyte Growth Factor (HGF), and a ligand of the Met-related receptor MST1-Receptor (MST1R/RON). Hepatocyte growth factor is a key mediator of invasive growth during embryonic development and is a potent angiogenic protein. The macrophage-stimulating protein pathway has recently been shown to promote metastasis in a mouse model of human breast cancer.

Conclusions: Recent advances in mass spectroscopy and data mining have enabled the reliable assay of the serum proteome. The "angiogenic switch" responsible for awakening dormant micrometastases may be a member of the macrophage-stimulating protein pathway. If additional experiments continue to implicate these proteins, their action may be able to be blocked by the administration of a peri-operative dose of a tyrosine kinase inhibitor specific for the MST1-Receptor.

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P37-13: MECHANISMS OF BREAST CANCER BRAIN METASTASIS

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Brain metastases are among the most feared complications in breast cancer that currently cannot be cured. Successful prevention and treatment will require that we know which mechanisms allow breast cancer cells to enter the brain and thrive within the central nervous system.

We established new human cell models to study breast cancer brain metastasis, and we follow establishment and progression of the disease as well as response to treatment in immune deficient mice by non invasive bioluminescence imaging. Our systems allow us to analyze mechanisms that circulating breast cancer cells use to penetrate the blood brain barrier and to survive and disseminate within the brain tissue.

Functional analyses and large-scale proteomic profiling of brain homing breast cancer cells revealed that the tumor cells use distinct functional pathways to enter the brain and to grow within the brain microenvironment. For example, expression of the adhesion receptor integrin $\alpha v \beta 3$ in an activated, high-affinity form promotes tumor cell brain colonization, and Her-2 strongly supports growth within the brain tissue. Importantly, breast cancer cell survival and expansion within the central nervous system is accompanied by significant changes in tumor cell metabolism characterized by oxidative pathways for energy production and simultaneous upregulation of antioxidant pathways that allow the tumor cells to detoxify reactive oxygen species generated during high-efficiency, oxidative glucose turnover. These metabolic changes reflect an adaptation of the tumor cells to metabolic energy pathways that normal brain cells use as well.

Thus, a subset of tumor cells may evolve during malignant breast cancer progression that is capable of gaining entry into the protected environment of the brain and which can differentiate along pathways that allow the tumor cells to thrive within the brain tissue. Our study raises the possibility of targeting distinct tumor cell properties for prevention and treatment of breast cancer brain metastasis.

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P37-14: THE CHEMOKINE RECEPTOR CXCR3 IS A DETERMINANT OF BREAST CANCER METASTASIS

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Breast tumor cell lines express several chemokine receptors including CXCR3, a G protein-coupled receptor that binds the ligands CXCL9, CXCL10, and CXCL11. We tested the hypothesis that malignant mammary epithelial cells employ CXCR3 to migrate to sites of ligand expression and that inhibition of either CXCR3 expression or receptor-signaling would inhibit the ability of breast tumor cells to metastasize. Using a panel of human breast tumors for which long-term survival was known, we now show that early-stage breast cancers frequently express CXCR3 and the level of expression is positively associated with poorer overall survival. In line 66.1 cells, a murine model of triple-negative (Her-2, ER, PR negative) metastatic breast cancer, we stably inhibited CXCR3 protein and mRNA and showed that migration of these tumor cells to specific CXCR3 ligands was compromised. When these 66.1-shCXCR3 cells were implanted into the mammary fat pad of syngeneic Balb/cByJ mice, spontaneous metastasis to the lung was markedly reduced by 72-94%, in comparison to vector-control cells. Gene silencing of CXCR3 had no impact on the size of the mammary fat pad-injected tumor. We also compared CXCR3 expression and function on an immortalized normal mammary epithelial cell line, Eph4, to malignant cells. Interestingly, normal mammary epithelium also expresses CXCR3, but the cellular responses are different in malignant versus normal cells. CXCR3 mediates migration of malignant cells to CXCR3 ligands, but normal cells are not stimulated to migrate. In contrast, proliferation of normal cells is inhibited by CXCR3 activation, but malignant cells are largely unaffected. AKT signal transduction pathway activation also differs in these two populations. These studies indicate that CXCR3 contributes to metastatic potential in a model of metastatic breast cancer. The positive association of CXCR3 and poor overall survival in women with breast cancer supports the further examination of this receptor as a therapeutic target.

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P37-15: APOPTOTIC RESISTANCE PROMOTES DORMANT TUMOR SPREAD BY IMPROVING THE SURVIVAL OF CIRCULATING TUMOR CELLS IN THE LUNG AND INCREASING THE PERSISTENCE OF AGGRESSIVE CYTOSKELETAL RESPONSES TO DETACHMENT

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Nearly 90% of all human solid tumors arise as carcinomas from epithelial cells. When these tumor cells begin to spread through the bloodstream, they cannot easily fit through capillaries due to the large size of epithelial cells. For this reason, epithelial tumor cells often become trapped in the first capillary bed they encounter. For circulating breast tumor cells, this first capillary bed is in the lung. We have shown previously that apoptotically resistant mammary epithelial cells can promote tumor dormancy by surviving the challenges of dissemination but failing to grow into tumors. In this study, we directly tested whether apoptotic resistance could promote increased survival of circulating breast tumor cells trapped in lung capillaries. Using in vivo bioluminescence imaging, we quantitate levels of dormant tumor cells in the lungs of living mice. While apoptotic resistance increases survival of these cells in the lungs, many apoptotically sensitive cells also survive long term. Such dormant survival in distant tissues establishes conditions allowing the independent evolution of primary and metastatic tumors. Preliminary studies on an inducible gene expression system and active whole-animal monitoring of tumor cell division are also reported. As a consequence of our focus on detached epithelial cells, we also observed novel cytoskeletal dynamics in these cells during this study. Detached cells produce unique protrusions of stabilized microtubules that promote cellular reattachment. Compelling data from intravital microscopy of tumor cells circulating in living mice supports a role for these microtubule protrusions in the initial attachment of bloodborne tumor cells to vessel walls during extravasation. We have extended these observations to human breast tumor cell lines and primary mouse mammary epithelial cells. Our data support a model in which the extension of microtubule protrusions represents a normal response of epithelial cells to detachment, rather than the result of a specific genetic alteration. However, when apoptosis is compromised, such microtubule protrusions persist for much longer periods. Together, these results support a model in which apoptotic resistance promotes dormant tumor cell dissemination by both increasing survival and the opportunity for invasive cellular responses. The eventual reemergence of these dormant cells as metastatic tumors is a major cause of patient death and novel therapies are needed to prevent the survival and dissemination of dormant tumor cells.

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P37-16: MIMICKING ONCOGENE-DEPENDENT TUMORIGENESIS IN AN IN VITRO, THREE DIMENSIONAL COLONY ASSAY

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Several genetic lesions are known to induce mammary tumors, and the affected signaling pathways have been extensively analyzed. However, little is known about how the genetic aberrations found in mammary epithelial cancers contribute to the histological phenotypes observed in vivo. Three-dimensional (3D) collagen/matrigel culture systems represent one means to study the impact of oncogenic mutations on specific aspects of the neoplastic phenotype, such as loss of cell polarization and sprouting, since 3D culture systems can recapitulate organotypic growth with respect to polarized morphology, specialized cell-cell contacts, and attachment to an underlying basement membrane. All of these features are necessary for the proper control of cellular proliferation, survival, differentiation, and secretion of soluble factors.

Efforts to develop 3D culture systems to study neoplasia have generally utilized immortalized cell lines (e.g., MCF10A cells; Debnath et al., 2005). We have now established conditions for cultivation of mammary gland-derived normal, hyperplastic, and tumorigenic cells in 3D basement membrane-extract gels. Cells derived from normal and hyperplastic mammary tissue grow to form hollow acinar structures while tumorigenic cells form enlarged solid structures (tumor cells from MMTV-Wnt1-transgenic mice) or irregularly shaped, enlarged hollow structures (tumor cells from MMTV-Neu-transgenic mice).

We have used these culture conditions to test the effect of inducible oncogenes on proliferation, polarized organization, and tubular sprouting of established polarized acini. We seeded the gels with primary mammary cells derived from tri-transgenic mice (TetO-Myc/TetO-KrasG12D/MMTVrtTA) that carry two doxycycline-inducible oncogenes. Within 6 days of doxycycline induction in the intact animal, the tri-transgenic mice form tumors that regress rapidly upon doxycycline withdrawal.

As expected, when seeded in 3D cultures, uninduced mammary cells from these animals form hollow acinar structures similar to structures formed by cells derived from nontransgenic animals. These structures are polarized as judged by the appropriate distribution of basal ($\alpha 6$ -integrin), baso-lateral (ECadherin), tight junction (ZO1), and apical (GM130) markers seen with whole-mount immunofluorescence. When induced in culture with doxycycline (1 μ g/ml) to overexpress Myc and mutant Kras, the structures grow into solid spheres within 4 days. When we elute doxycycline from the gels to deinduce Myc and Kras, growth ceases and the inner region of the solid clusters of cells clears. Induced solid structures maintained on doxycycline during this period (10d) do not show any changes in the inner mass of cells. A more detailed analysis of the response to doxycycline withdrawal, employing time-lapse microscopy and histological sections, reveals that cells in the center lose mitochondrial polarization and activate Caspase 3, beginning 20 hours after removal of the drug. The outermost layer of cells remains viable and retains the irregular shape assumed during doxycycline stimulation. Upon renewed exposure to doxycycline, these remaining hollow structures fill in, suggesting the outer layer of protected cells to be a correlate of dormant tumor cells. We are currently analyzing other features of these cells and following the effects of inducing Myc or Kras oncogenes individually.

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BONE METASTASIS

Poster Session P38

P38-1: HIGH BONE TURNOVER MAY INCREASE BREAST CANCER METASTASIS TO BONE

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Tamoxifen is bone sparing for postmenopausal women, but its use in breast cancer is being rapidly superseded by aromatase inhibitors (AIs). Unlike Tamoxifen, AI therapy for breast cancer results in high bone turnover, which can lead to osteoporosis. Cancer treatment-induced bone loss is likely to become the most common skeletal complication of malignancy. Our hypothesis is that breast cancer bone metastases are increased when bone is in a state of high turnover from estrogen deficiency. We developed a mouse model of high bone turnover from estrogen deficiency to study its effect on breast cancer metastasis to bone.

To induce high bone turnover, 4-week-old female athymic mice were randomized to ovariectomy (OVX), sham surgery (sham), treatment with the AI Letrozole (125 mcg/sc/d), or treatment with vehicle. Bone mineral density (BMD), as assessed by Lunar PIXImus, was done at baseline and then every 2 weeks. Mice were sacrificed 28 weeks after surgery and 16 weeks after treatment with Letrozole or vehicle. OVX mice had significantly decreased uterine weight ($P < 0.0001$) compared to sham mice thus confirming estrogen depletion. OVX mice had increased body weight ($P < 0.0001$) and fat mass ($P < 0.0001$) and decreased BMD at the total body ($P = 0.0257$), spine ($P < 0.0001$), and tibia ($P < 0.0001$) compared to sham mice. BMD did not significantly differ between OVX and sham mice at the femur. Letrozole mice also had decreased uterine weight ($P = 0.0012$) compared to vehicle. As with OVX, Letrozole mice had increased body weight ($P < 0.0001$) and fat mass ($P < 0.0001$) compared to vehicle. However, in contrast to OVX, Letrozole mice had increased BMD at the total body ($P < 0.0001$) and tibia ($P = 0.0002$) compared to vehicle. There was no difference in BMD at the spine or femur between Letrozole and vehicle mice.

Histomorphometry showed no difference in trabecular bone volume at the femur or tibia when comparing OVX to sham surgery or Letrozole to vehicle. However, bone marrow cultures from OVX mice exhibited a greater number of colony forming unit (CFU) fibroblasts ($P < 0.0001$), CFU osteoblasts ($P < 0.0001$), and TRAP (+) osteoclasts ($P = 0.0002$). Bone marrow cultures from Letrozole mice also exhibited a greater number of CFU fibroblasts ($P = 0.0001$), CFU osteoblasts ($P < 0.0001$), and TRAP (+) osteoclasts ($P = 0.0047$) compared to vehicle. These data indicate a high bone turnover state in both OVX and Letrozole mice as compared to the control mice.

In another experiment, 4-week-old female nude mice were randomized to OVX, sham surgery, or to daily treatment with Letrozole or vehicle and then subsequently underwent inoculation with the estrogen receptor-negative human breast cancer cell line MDA-MB-231 via intracardiac injection. Mice were x-rayed at baseline and then at 1-week intervals to monitor for development and progression of bone metastases. Interestingly, total body x-ray lesion area was greater in sham mice compared to OVX ($P < 0.0001$), but there was no difference in total body lesion area between the Letrozole and vehicle mice.

A high bone turnover state is induced by estrogen deficiency from breast cancer therapy. However, OVX and Letrozole had different effects on bone mass. Further studies are needed to characterize the effects of OVX and AI therapy on bone. OVX mice were protected from breast cancer metastasis to bone. The reason for this finding is unclear but will be the topic of further investigation.

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P38-2: NOTCH3 – JAGGED1 EXPRESSION REGULATES TGF β -DEPENDENT ANCHORAGE-INDEPENDENT GROWTH AND BONE METASTASIS OF BREAST CANCER CELLS

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Bone is the preferred organ for breast cancer metastasis. We address the Notch signaling interactions between breast cancer cells and bone marrow stromal osteoblasts (BMSO) that we hypothesize to be important for metastasis. Notch signaling initiated by BMSO was recently shown to be critical for the engraftment and sustenance of normal long-term hematopoietic stem cells, and cancer cells are found to gain metastatic advantage through similar bone marrow interactions. Our goal is to define the Notch signaling components supporting breast cancer that are initiated by BMSO and analyze the response pathways. Bone-derived TGF β plays major roles in the vicious cycle of bone destruction and development of bone metastases. Using human breast cancer cells, MDA-MET (a bone-metastatic subline of MDA-MB231) and MDA-MB-435, we observed that TGF β 1 at 1 ng/mL stimulated the mRNA expression of Notch3 (2.7-fold) and Jagged1 (2.2-fold), as well as expression of the Notch transcriptional target Hes1 (6.1-fold). Enhanced protein expression of Jagged1 in response to TGF β 1 was also detected. In a soft agar overlay assay, TGF β 1 stimulated the anchorage-independent colony growth of breast cancer cells by 2.7-fold over control cells. To test the role of Notch3 in tumorigenesis, we employed shRNA stable interference to generate a clonal

subline (MDA-MET-shN3) with decreased mRNA level of Notch3. This cell model also exhibited a dramatic reduction in Jagged1 protein expression, whereas Notch1 remained unchanged. Decreased Notch3/Jagged1 expression in MDA-MET-shN3 cells inhibited the TGF β -stimulated anchorage-independent growth by 88% and significantly inhibited the TGF β -stimulated phosphorylation of Smad3; phosphorylation of Smad2 was also slightly reduced. Similar to TGF β treatment, coculture of MDA-MET and MDA-MB435 with differentiated human bone marrow stromal osteoblasts (hBMSO) upregulated the mRNA levels of Notch3 (3.5-fold) and Jagged1 (2.2-fold). The osteoblasts also stimulated anchorage-independent tumorigenic colony formation, and this was inhibited by L685,458, a γ -secretase inhibitor that blocks Notch signaling. Furthermore, anti-TGF β blocking antibody at 1 μ g/mL completely inhibited osteoblast-stimulated anchorage-independent growth in breast cancer cells. To test the role of Notch3 in tumorigenesis in vivo, we studied bone metastasis by intracardiac injection in nude mice. Compared with robust osteolytic metastasis by control empty plasmid-transfected cells, inoculation of MDA-MET-shN3 cells with constitutively reduced Notch3/Jagged1 expression markedly decreased the extent of osteolytic lesions. Taken together, our results suggest the enhanced Notch3/Jagged1 expression in breast cancer cells, triggered by TGF β originating from osteoblasts in the bone marrow niche, may stand as a novel mechanism for regulating bone metastasis. Elevated expression and signaling of Notch and its ligands in breast cancer are correlated with poor prognosis. That Notch3 signaling may also control the fate of putative stem cells in breast cancer requires further analysis of the TGF β -responsive tumorigenic cell phenotype.

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P38-3: COX2 EXPRESSION CORRELATES WITH BONE MARROW MICROMETASTASES IN PATIENTS WITH STAGE I–III BREAST CANCER

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Background and Objectives: COX2 expression has been associated with different stages of breast cancer progression. Our preclinical studies, involving analysis of bone-seeking clones (BSCs) isolated from a xenograft mouse model, have provided strong evidence that COX2 is important for bone metastasis. The BSCs produce a higher level of PGE₂, uPA, IL-8, and/or IL-11 than the parental cells originally injected into the mice, and a COX2 inhibitor halted bone metastasis. Bone marrow micrometastases (BMM) found in patients at time of diagnosis have been documented to correlate with subsequent clinical metastases and worse outcome. We hypothesized that increased COX2 expression in primary human breast cancers is responsible for BMM.

Methods: Clinical and pathological data were collected from 109 patients with operable breast cancer enrolled in a prospective clinical study comparing primary tumor characteristics from tumor tissue collected at surgery for primary breast cancer (Stage I–III) with BMM. Primary tumors were immunostained with a monoclonal antibody directed against COX2. Positive COX2 status was determined as 5% or more of tumor cells staining positive. Bone marrow aspirates were obtained from bilateral iliac crests just prior to removal of the primary tumor. A mixture of anti-cytokeratin antibodies was used to immunostain BMM. Detection of one or more cytokeratin-positive cells was considered a positive result for BMM. Primary tumor characteristics studied included: tumor size, ER, PgR, HER2, tumor grade, histological type, lymphovascular invasion, Ki-67, and lymph node metastasis. Statistical analysis utilized Chi squared tests.

Results: Currently, we have assessed 83 primary tumor specimens for COX2. We found that COX2 expression in primary breast cancer correlated with BMM in a highly statistically significant manner ($p = 0.001$). Statistical analyses of correlations of COX2 positivity in primary tumor with other clinically relevant indicators revealed a correlation with triple-negative status of cancers, defined by lack of estrogen receptor, progesterone receptor, and HER2 expression/amplification ($p < 0.0001$). This result is significant given the lack of therapeutic options in these patients. Triple-negative status was present in 29% (32/109) and COX2 expression was present in 29% (24/93) of patients. Patients with triple receptor-negative status expressed COX2 more frequently than patients expressing any one receptor ($p = 0.001$, OR = 5.79). Significant correlations were also obtained between triple receptor-negative status and grade of tumor ($p < 0.001$, OR = 6.53) and Ki-67 ($p = 0.01$, OR = 6.43).

Conclusions: COX2 produced in primary breast cancer cells may be vital to the initial development of BMM, subsequently leading to osteolytic bone metastases in breast cancer patients. Triple-negative primary breast tumors express high levels of COX2 as compared to tumors expressing any one of the three primary tumor markers. Identification of patients expressing increased COX2 could open a potential avenue of targeted treatment using COX2 inhibitors. This is especially important in triple receptor-negative patients where targeted therapies are very limited.

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P38-4: COMBINED IN VITRO AND IN VIVO STRATEGIES TO ISOLATE BONE METASTATIC BREAST TUMOR VARIANTS IDENTIFY LAMININ-511 AS A POTENT PRO-METASTATIC SUBSTRATE

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Background and Objectives: Changes in the expression of specific laminin (LM) isoforms have been associated with tumor progression. In particular, increased vascular^{1,2} and tumor cell² expression of LM-511 is correlated with breast tumor progression and metastasis. Similar correlative evidence has been reported in prostate³ and non-small cell lung carcinomas⁴. Consistent with a potential role in breast cancer progression, we recently showed that LM-511 promotes migration and invasion of metastatic mouse and human breast tumor cells in vitro². Whereas these studies support a role for LM-511 in breast cancer metastasis, direct evidence that LM-511 contributes to metastasis in vivo is still lacking. Therefore, the overall objective of this study was to demonstrate that the ability of breast tumor cells to interact with and respond to LM-511 regulates their metastatic potential in vivo.

Methodology: Subpopulations of cells were isolated from the weak lung metastatic 4T1 cells based on their ability to rapidly migrate toward LM-511 in vitro. After four rounds of enrichment in vitro, the resulting variants (4T1LMF4) were inoculated into the mammary fatpad of syngeneic Balb/c mice. Bone metastatic nodules were isolated, expanded in culture, and this process repeated to enrich for bone metastatic cells (4T1LMF4/BM2). Parental 4T1 cells and variants were characterized functionally in standard in vitro assays. Gelatinase expression was analyzed by gelatin zymography, and changes in integrin receptor expression were determined by flow cytometry. Differential activation of signaling pathways (AKT, FAK, and ERK-1/2) in 4T1 variants in response to LM-511 and/or bone-derived soluble factors was analyzed by immunoblotting. Metastatic spread in vivo was evaluated semi-quantitatively by visual inspection and histology.

Results: LM-511-selected 4T1LMF4 variants displayed dramatically enhanced metastasis to several organs, most notably to bone, compared to parental 4T1 cells following orthotopic injection in Balb/c mice. Their metastatic potential was further enhanced through serial in vivo selection of bone metastatic cells (4T1LMF4/BM2). Increased bone metastasis in 4T1LMF4/BM2 variants was characterized by changes in integrin receptor expression, rounding of the cells on LM-511, and re-establishment of stress fibers in the presence of both LM-511 and serum-free bone conditioned medium. These changes were accompanied by enhanced migration and induction of MMP-9 expression in response to bone-derived soluble factors alone or synergistically with LM-511 compared to 4T1 cells. Whereas both LM-511 and bone-conditioned medium induced potent and prolonged activation of ERK-1/2 in 4T1LMF4/BM2 cells, AKT was selectively activated by bone-conditioned medium.

Conclusions: The results above provide strong evidence that responsiveness to LM-511 is a critical determinant dictating the metastatic potential of breast tumors in vivo. Cooperation between LM-511 and bone-soluble factors suggests that targeting of these interactions may represent novel antimetastatic targets for the treatment of advanced breast cancer patients.

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P38-5: STUDY THE INFLUENCE OF ARTHRITIS ON BREAST CANCER-ASSOCIATED BONE METASTASIS

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The preference of breast cancer cells for bone as a metastatic site is underscored by the fact that 65%–75% of patients with advanced disease develop bone metastases. We hypothesize that chronic inflammatory milieu and osteoclastic bone resorption caused by autoimmune arthritis may influence the recruitment, retention, and proliferation of tumor cells in the bone and promote metastasis. Our aims are to evaluate (1) the incidence of bone metastasis in mouse models of spontaneous breast cancer that are induced to develop autoimmune arthritis; (2) the incidence of bone metastasis in a spontaneous model of autoimmune arthritis that are induced to develop breast carcinomas; (3) the efficacy of anti-IL-17 antibody (IL-17 is a crucial cytokine or osteoclastic bone resorption in autoimmune arthritis patients) treatment alone, or in combination with other anti-inflammatory agents in the prevention of bone metastasis; and (4) the incidence of arthritis in breast cancer patients with or without bone metastasis.

Results: Since the receipt of the award in October 2007, we have generated preliminary data on the first two aims of the project. When the PyV MT mice that develop spontaneous breast cancer were injected intradermally with 2 mg/mL Type II collagen to induce arthritis, we observed a 2–3-fold increase in epithelial metastatic cells in the bone marrow of these mice compared to control mice. This was a clear indication of epithelial cell recruitment into the bone microenvironment during breast cancer progression. We noted increased levels of CD11b⁺/Gr1⁺ positive myeloid suppressor cells (MSCs) in the bone marrow of the PyV MT mice injected with the collagen compared to PyV MT mice without the collagen. Within the tumor microenvironment, the T regulatory cells (Tregs; CD4⁺/CD25⁺/FOXP3⁺) were greatly increased with collagen injection compared to PyV MT mice without collagen. The MSCs and Tregs are both associated with immune tolerant milieu conducive for tumor cells to survive and proliferate rapidly. Of importance is that the PyV MT mice treated with collagen had bigger primary tumors and higher frequency of lung metastasis compared to PyV MT mice that did not receive collagen. For the second aim, we have generated the baseline data with a high and low metastatic breast cancer cell lines; namely, the 4T1 and the TUBO cells in an immune competent Balb/c mice. These experiments are now being repeated in the SKG mice that develop spontaneous autoimmune arthritis. The 4T1 and TUBO tumor cells were injected in the mammary fat pad. Fifteen days post-challenge; there was a 2-fold increase in the levels of epithelial metastatic cells in bone marrow of 4T1-challenged mice compared to TUBO-challenged or non-tumor-bearing mice. This was associated with lower NK cells and higher Tregs within the tumor microenvironment of the 4T1 tumor compared to the TUBO tumor. In contrast to the PyV MT mice, we observed a decrease in the MSCs in the 4T1- and the TUBO-challenged mice. However, the B7H4 positive cells (another form of suppressor DCs) were higher in the 4T1-challenged mice suggesting that the MSCs may not be a main player in the injectable tumor models and that other cell types may be associated with the immune tolerant milieu. The data so far are promising and if the proposed study is conclusive, it could be used in the prevention of bone metastasis, in combination drug delivery, or as a risk-assessment tool for possible future bone metastasis.

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P38-6: A ROLE OF MEPE-OF45 IN BONE METASTASIS FROM BREAST CANCER

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Bone metastasis frequently occurs in up to 70% of patients with advanced breast cancer, which is one of the most severe threats for breast cancer patient survival. Osteolysis is one of the essential events during the multistep process of bone metastasis from breast cancer. However, it remains unclear what kind of osteolysis factors play a key role in bone metastasis from breast cancer. Matrix extracellular phosphoglycoprotein/osteoblast/osteocyte factor 45 (MEPE/OF45) that is involved in osteolysis was first identified in human bone-related tissue in 2000, and identification of the homologue of MEPE/OF45 in rat and mice then followed. Although since MEPE/OF45 was cloned, its function related to bone metabolism has been widely studied. There are no studies reporting any connection between MEPE/OF45 and bone metabolism from breast cancer. Here we show that MEPE/OF45 is higher expressed in the breast cancer cell lines with high bone metastasis frequencies than that in the breast cancer cell lines with low bone metastasis frequencies derived from both human and mouse tumors. These phenotypes suggest that MEPE/OF45 might be involved in bone metastasis of breast cancers. To test this hypothesis, we are currently establishing the cell lines that stably express siRNA of MEPE/OF45 in both human and mouse breast cancer cells with high bone metastasis frequencies. We will compare the metastasis features in vitro by examining the migration, adhesion, and invasion property of breast cancer cells (from both human and mouse) with and without MEPE/OF45 siRNA expression. We will also compare bone metastasis frequencies in vivo by examining the bone metastasis frequencies from a xenograft of breast cancer cells with and without MEPE/OF45 siRNA expression in both MEPE/OF45^{+/+} mice and MEPE/OF45^{-/-} mice. The results from these experiments will reveal whether and how the highly expressed MEPE/OF45 in breast cancer cells is linked to their high bone metastasis feature. We believe that these results will identify a new target for preventing bone metastasis from breast cancer, which will potentially benefit treatment for breast cancer patients.

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P38-7: NOVEL MECHANISM OF BREAST CANCER-INDUCED OSTEOLYSIS

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Background and Objectives: Breast cancer (BC) frequently metastasizes to bone, resulting in several serious complications and poor prognosis. BC-induced osteolysis is not only a consequence but, more devastatingly, also a cause of BC skeletal metastasis.

Three key players, osteoclasts (OC), BC cells, and osteoblasts (OB), are involved in BC-induced osteolysis. Briefly, BC cells express PTHrP, which stimulates the production of RANKL by OB. The produced RANKL promotes the differentiation of OC precursors into mature OC to resorb bone, resulting in the release and activation of TGF β embedded in bone matrix. The released TGF β further stimulates the expression of PTHrP. As such, bone resorption is augmented by the vicious cycle. Although this mode of action is well established, it is unclear how BC cells and OB are brought to the scene to act. It has been proposed that BC cells are attracted to the resorption sites by the released factors in the tumor-induced osteolysis. However, this notion creates a chicken and egg paradox of which comes first, the BC cells/OB recruitment or the released factors from bone by OC. Specifically, in tumor-induced osteolysis, before the start of bone resorption, OB and BC cells must be recruited to bone surface to produce RANKL, which is required for OC formation. However, based on the current belief, these cells are attracted to the sites through factors released from bone matrix by OC, but how could OC be formed in the first place? We propose a new hypothesis that OC precursors play a key role in OB and BC recruitment to bone. Our objective was to determine whether OC precursors play a role in recruiting BC cells and OB onto the bone in vitro.

Methodologies: We performed chemotaxis assays with the following conditioned media (CM): CM1 – from OC precursors cultured in Teflon beaker, CM2—from OC precursors cultured in tissue culture plates without bone slices, and CM3—from OC precursors cultured in tissue culture plates with bone slices. Note that the CM were all prepared using MEM containing 0.2% FBS. The control medium is MEM containing 0.2% FBS. A total of 600 μ L CM was added to one well in Transwell. BC cells (MDA-MB-231) or OB (2×10^6 /mL) were in MEM with 0.2% FBS. Transwell were inserted into well, and 100 μ L cells (2×10^5 each insert) were added to the inside compartment. Transwell were cultured for 4 hours at 37°C and 5% CO $_2$. After 4 hours, cells were fixed for 30 seconds. Cells were stained for 1 hour with Crystal Blue and then counted.

Results: All three CM (CM 1, CM2, and CM3) exhibited chemotaxis on MDA-MB-231 cells compared with control medium. More importantly, CM3 demonstrated a higher capacity in inducing chemotaxis on MDA-MB-231 cells than CM1 and CM2. Moreover, all three CM (CM 1, CM2, and CM3) exhibited chemotaxis on OB compared with control medium. However, these 3 CM showed no significant difference in their ability to induce chemotaxis on osteoblasts.

Conclusions: OC precursors can exert chemotaxis on BC cells and OB, suggesting that OC precursors play a key role in BC bone metastasis by recruiting OB and BC cells to the active bone remodeling sites. Importantly, these studies have fostered a new direction for investigation, which will not only lead to elucidation of the precise mechanism underlying BC cells/OB recruitment to bone but may also reveal more effective and specific therapeutic strategies for treating and preventing BC bone metastases.

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P38-8: HYPOXIC RESPONSE OF BREAST CANCER IS INDUCED BY RANKL: RELEVANCE TO BONE METASTASIS

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Hypoxia in malignant disease is linked to genomic instability, invasion, metastasis, tumor angiogenesis, and therapeutic resistance. Hypoxic tumor cells accumulate hypoxia inducible factor-1 α (HIF-1 α), causing tumor progression by transcriptional activation of hundreds of genes with hypoxia response elements (HRE) in their promoters: glucose transport, glycolysis, survival, and angiogenesis. Breast cancer cells employ poorly understood mechanisms to stabilize HIF-1 α and/or upregulate its expression, leading to HIF-activated gene expression and malignantly advantageous aerobic glycolysis ("Warburg Effect"). Elevated HIF-1 α levels promote dedifferentiation and tumor progression in DCIS. Lymph node-positive breast cancer with elevated HIF-1 α has a poor prognosis. Accordingly, we have explored breast cancer hypoxia in the context of the metastasis of breast cancer to its favored site—bone. A unique (RANKL-RANK) ligand-receptor interaction between osteoblast/bone marrow stromal cells and metastatic breast cancer cells is hypothesized to cause stabilization of HIF-1 α and promote tumor progression. RANK (receptor activator of NF- κ B) is constitutively expressed by breast cancer cells, and RANKL (RANKL) is expressed by osteoblasts where it normally serves as the required stimulus for osteoclast formation and activation. HIF prolyl hydroxylases (PHDs) require oxygen to convert Pro-564 and Pro-402 to hydroxyprolyl residues, which targets HIF-1 α for VHL binding and proteasome-mediated destruction. Low concentrations of molecular oxygen inhibit PHD2, thus stabilizing HIF-1 α . When MDA-MB231 human breast cancer cells were treated with RANKL, immunoblotting showed HIF-1 α and HIF-2 α stabilization in direct proportion to the length of exposure to RANKL. RANKL also caused time-dependent increases in the protein levels of PHD2, PHD3, and PHD4, suggesting a possible feedback mechanism for suppression of the hypoxic response. Similar responses also occurred in the presence of 0.2 mM CoCl $_2$ that induces hypoxic conditions by PHD inhibition. Silencing of HIF-1 α with siRNA caused compensatory upregulation of HIF-2 α , (9-fold) while reducing expression of PGK1, COX2, PHD3, and VHL. With HIF-

1 α silenced, HIF-2 α was upregulated by RANKL (3.8-fold) while other genes were slightly upregulated (endoglin, SDC4, PLAUR, and MMP2). This is consistent with differential gene regulation in breast cancer cells by HIF-1 α versus HIF-2 α . Phosphospecific protein immunoblotting revealed that RANKL-RANK signal transduction in MDA-MB231 involves several pathways (ERK1/2, PI3K-Akt, and p38MAPK), thus linking one or more of these cascades to the observed hypoxic response involving HIF stabilization. In conclusion, osteoblast-cancer cell-cell interactions that facilitate RANKL-RANK signaling of metastatic breast cancer in the bone microenvironment cause activation of the hypoxia pathway in breast cancer. Inhibition of this signaling may be key to unraveling and interfering with the complex process of bone metastasis.

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P38-9: ErbB2 OVER-EXPRESSION IN BREAST CANCER BONE METASTASES: INHIBITION OF HEMATOPOIESIS

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A common site of metastasis in breast cancer is the bone. Cancer cells growing in the bone matrix cause rapid bone destruction, pain, and fractures. In addition, recent studies have shown that metastases within the marrow may disrupt hematopoiesis by displacing or killing progenitor cells thus leading to anemia, neutropenia, and thrombocytopenia, common to metastatic cancer patients. A secreted cellular protein, NGAL (neutrophil gelatinase associated lipocalin) has been shown to be produced by leukemia cells resulting in death of hematopoietic cells and halting hematopoiesis. NGAL production in leukemia cells appears to be regulated by tyrosine kinases. ErbB2 (also known as HER2) is a receptor tyrosine kinase over-expressed in 20%–30% of invasive breast carcinomas as well as other cancer types. ErbB2 over-expression has been shown to correlate with poor prognosis and increased incidence of metastasis. It has also been shown that bone marrow micrometastases express more ErbB2 than their primary breast tumor, suggesting a selection bias for ErbB2 over-expressing cells in the bone microenvironment. Excitingly, we have found that ErbB2-positive breast cancer cells also secrete NGAL. Therefore, we hypothesized that ErbB2 over-expression in breast cancer cells leads to increased NGAL expression, resulting in impaired hematopoiesis and increased tumor growth in the bone.

We collected conditioned media from ErbB2 over-expressing breast cancer transfectants and parental control ErbB2 low-expressing cells. We added the conditioned media onto erythroblasts and examined the effect on erythroblast survival. Conditioned media from ErbB2 over-expressing cells caused an increase of apoptosis of erythroblasts when compared to the conditioned media from parental control cells. Multiple experiments also showed that NGAL secretion, erythroblast cell death, and ErbB2 expression levels are dependent on one another. These results indicated that ErbB2 over-expression results in increased NGAL production, hence leading to hematopoietic cell death.

We examined the hypothesis in vivo using the MMTV-neu transgenic mouse model. After MMTV-neu mice developed breast tumors, they presented anemia consistent with bone marrow failure. We then sought to elucidate the mechanism of ErbB2-mediated NGAL secretion. ErbB2 over-expression resulted in increased NGAL mRNA levels due to transcriptional upregulation. NGAL promoter deletion experiments identified that 267bp NGAL promoter upstream of transcription initiation site is required for ErbB2-mediated upregulation of NGAL. This fragment contained potential NF- κ B, STAT3, and C/EBP1 consensus binding sites. We showed that ErbB2 over-expression resulted in increased STAT3 activity and activation of NF- κ B. Currently we are using ChIP assay to determine which transcription factor is involved in the ErbB2-mediated NGAL upregulation.

These novel findings may identify opportunities for pathway intervention and ultimately decrease hematopoietic cell death in ErbB2 over-expressing breast cancer bone metastasis. Understanding the role of ErbB2 and NGAL in the inhibition of hematopoiesis in breast cancer bone metastasis may allow development of prognostic tests and preventative therapy thereby decreasing bone destruction and significantly impacting the management of bone metastasis in breast cancer patients.

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P38-10: TREATMENT STRATEGY TO REVERSE OSTEOLYTIC BREAST CANCER METASTASIS USING OSTEOBLASTS

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Rationale: The most prevalent site of breast cancer metastasis is bone. The majority of bone metastases are osteolytic and by the time osteolysis is detected, >50% of the bone matrix is lost. Using bone metastatic xenografts, we found that in addition to activating osteoclasts (OC), metastatic cells eliminated osteoblasts (OB) via apoptosis. Our finding may explain failure of bisphosphonate-treated patients to repair osteolytic lesions.

Hypothesis: Replacement of eliminated osteoblasts would re-set the OB:OC balance so that lost bone matrix can be replaced.

Primary Objective: To provide proof-of-principle evidence that a mesenchymal stem cell-based therapy could replace OB lost during the formation of osteolytic breast metastases.

Relevance: Repair of already existing bone lesions would benefit patients whose disease progression has been halted using conventional therapies.

Approach: We will utilize pre-OB cells that home to bone, differentiate, deposit, and calcify matrix. Murine pre-OB cell line, MC3T3-E1, will be engineered to express mCherry using lentiviral expression so that detection will be easier in co-culture and in vivo experiments.

Aim 1: Will test homing and functionality of the red MC3T3-E1 (E1Red) cells at sites of osteolysis. Pilot experiments will determine seeding efficiency in the healthy femur. E1Red will be injected into athymic mice and their presence and function in bone will be monitored using fluorescence microscopy and histomorphometry. Comparison of seeding in healthy versus osteolytic bone will be done. Mineralization will be monitored by observing regions containing E1Red commensurate with two time-spaced doses of autofluorescent tetracycline.

Aim 2: Since OB are eliminated by apoptosis, simple replacement would not be sufficient (i.e., the new OB would also be eliminated by residual tumor cells, if any). To overcome this limitation, we will over-express an anti-apoptotic molecule (initially Bcl2) in E1Red cells. Co-culture will confirm resistance of the OB to apoptosis as measured by TUNEL staining and caspase 3/7 activity.

Aim 3: Mice receiving intracardiac injection of GFP-tagged human breast tumor cells will also receive E1Red cells before or after tumor cell inoculation. Data from Aim 1 will determine optimum number of pre-OB cells to be injected. Kinetics of tumor cell arrival and growth in the bone (which are not expected to change) and extent of osteolysis (which is expected to decrease or no longer progress) will be quantified by histomorphometry and compared to non-tumor-bearing controls. An irrelevant cell line (NIH3T3) will be injected as a negative control.

Innovation: Metastatic breast carcinoma cells manipulate bone microenvironment to induce osteolysis. Most studies have focused on OC activation via the "vicious cycle" involving secretion of PTHrP or RANK ligand. The role of the OB in osteolytic breast lesions is understudied. Based upon our preliminary data and clinical observations that imply OB dysfunction, the proposed experiments will test, for the first time, experimental manipulation of OB number and function in osteolytic breast cancer metastasis.

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P38-11: A NEW IN VITRO MODEL OF BREAST CANCER COLONIZATION OF BONE

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Breast cancer frequently metastasizes to the skeleton where it causes bone loss with accompanying pain, hypercalcemia, and fractures. Bisphosphonates administered to inhibit osteoclast activity and slow lesion formation are not curative. We hypothesize, therefore, that cancer cell-mediated osteolysis is partly due to loss of functional osteoblasts (OB). To test this hypothesis, we used a specially designed bioreactor (BR) to study the interaction of metastatic, osteolytic breast cancer (BC) cells with a 3D OB tissue grown over extended culture periods. The aims are to (1) determine the effect of metastatic BC cells on the physiology of OB tissue; (2) determine how metastatic BC cells affect the morphology and organization of the OB tissue; and (3) test known stimulators/protectors of OB function in the presence and absence of BC cells as a means of blocking the destructive effects of cancer on bone-forming OB tissue.

MC3T3-E1, a murine preosteoblast line, was cultured both in standard tissue culture, polystyrene (TCP) plates, and in the BR, for various times, up to 31 days for TCP and up to 10 months for the BR. Human metastatic BC cells, MDA-MB-231^{GFP}, were added to the cultures for up to 7 days. In both TCP and 3-D culture, the OB proliferated and differentiated, as evidenced by the expression of type I collagen, osteocalcin (OCN), osteonectin (OSN), calcium deposition, and mineralization (von Kossa stain). In the BR, however, osteoblasts formed an osteoid-like tissue with 6-8 cell layers in a thick extracellular matrix. By contrast, only 1-2 cell layers formed in TCP. Additionally, bone nodules and chips formed in the BR but not in TCP and were found by energy-dispersive x-ray analyses and IR spectroscopy to contain calcium and phosphate consistent with bone. The 2-D cultures could be grown for only about 30 days before numerous apoptotic cells became evident, and the cells began to peel from the plate. In contrast, 3-D cultures have been successfully maintained for 10 months with apparently normal phenotype.

BC cells, added to OB grown in TCP and BR at 1:10, 1:100 or 1:1000 BC:OB, attached and colonized. More BC colonies formed in younger OB cultures and with greater BC inoculate at all stages of OB maturity. Addition of BC cells to the BR at about 30 to 60

days caused a dramatic change in OB morphology from cuboidal to spindle shaped. The BC cells aligned themselves with the OB in a pattern recapitulating cell filing observed in pathological tissue. Analysis by Z-stack sectioning revealed that the BC cells penetrated the OB tissue in the BR. Interestingly, only OB near the tumor cell colonies changed shape in TCP, and BC penetration of OB tissue was not evident. Analysis of the culture medium revealed that, in the presence of the BC cells, there was a decrease in differentiation proteins (OCN, type I collagen, OSN) and an increase in the inflammatory cytokines (IL-6 and MCP-1), consistent with downregulated collagen synthesis and a profound OB inflammatory response.

In summary, although substantial information can be obtained from both 2D and 3D cultures, the BR provides a more stable, long-term culture that mimics the in vivo environment to a greater extent than TCP. BR provides a model system that bridges the gap between TCP and animals that allows manipulation of the microenvironment as well as BCs.

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P38-12: THE ROLE OF ADRENOMEDULLIN IN BREAST CANCER BONE METASTASIS

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The majority of women with advanced breast cancer develop incurable bone metastases, which cause bone pain, skeletal fractures, hypercalcemia, nerve compression, and morbidity. Current antiresorptive treatments do not improve survival. Tumor-secreted factors are promising targets for treatment of bone metastasis. Such factors stimulate bone cells, which in turn secrete factors that stimulate the tumor cells, leading to an incurable vicious cycle. Adrenomedullin (AM), a 52 amino acid peptide is commonly secreted by breast cancer cells and stimulates osteoblasts in vivo and in vitro, making it a potential treatment target. AM overexpression in prostate cancer cells increased bone metastases in mice, while siRNA knockdown of AM in lung adenocarcinoma cells decreased bone lesions. We hypothesized that decreasing AM expression in breast cancer cells would decrease bone metastases.

MDA-MB-231 breast cancer cells were transfected with either a shRNA expression vector for AM or a control scrambled shRNA sequence. Single-cell AM knockdown and control shRNA clones were isolated. Knockdown was confirmed using RT-PCR. Clones were cultured for 60 days without antibiotic treatment and AM mRNA was re-analyzed by PCR to confirm clonal stability. Several clones were tested for expression of tumor-secreted factors by PCR. In vitro proliferation was measured by MTT assay. Two stable AM shRNA and two control shRNA clones were injected into the left cardiac ventricle of nude mice to form a bone metastasis mouse model. Bone lesions were monitored by x-ray. The same clones were also injected into the mammary fat pad of nude mice to assess changes in mammary fat pad tumor take and growth.

AM mRNA was stably knocked down 90% in MDA-MB-231 cells. AM knockdown clones had decreased IL-11 and ET-1 mRNA levels but no change in Cyr61, CTGF, IL-8, PTHrP, and IL-6 mRNA levels compared to control clones. Decreased IL-11 or ET-1 mRNAs were not rescued by adding exogenous AM, suggesting an intracrine action of AM. No significant cell morphological differences were noted by microscopy. Knockdown clones were more sensitive to growth inhibition by taxol in vitro than controls. The knockdown clones caused more total bone lesion area in mice by x-ray compared to control clones. The knockdown clones exhibited decreased tumor take and growth within the mammary fat pad.

In contrast to the results found in lung and prostate cancer bone metastasis studies, AM knockdown promotes bone metastases by osteolytic breast cancer cells. However, AM knockdown inhibits growth within the mammary fat pad, indicating that the tumor microenvironment may play a role in AM's function in breast cancer. Bone histomorphometry will assess changes in osteoclast and osteoblast numbers and tumor volume at bone metastatic sites in response to AM knockdown. Immunohistochemistry will assess changes in angiogenesis.

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P38-13: THE ROLE OF OSTEOBLAST-DERIVED CYTOKINES IN BONE METASTATIC BREAST CANCER

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Breast cancer is the second leading cause of cancer deaths among American women. In late stages of the disease, breast cancer preferentially metastasizes to the bone. While the mechanism for directional metastasis is unknown, the bone microenvironment likely

provides a fertile soil for metastatic breast cancer cells. In addition to affecting osteoblast and osteoclast properties, we have evidence that metastatic breast cancer cells create a unique bone microenvironment by co-opting osteoblasts to increase their production of inflammatory cytokines. These cytokines may be chemoattractants, growth, or maintenance factors for cancer cells as well as osteoclasts.

MC3T3-E1 murine osteoblasts were grown to 4 (growth), 10 (early differentiation), and 20 days (late differentiation), and incubated with cells or conditioned medium from human metastatic breast cancer MDA-MB-231 variants (parental MDA-MB-231-W, parental MDA-MB-231-PY, bone-seeking MDA-MB-231-BO, or brain-seeking MDA-MB-231-BR). Culture supernatants were assayed for cytokine expression with species-specific Bio-Rad Bio-Plex™ cytokine arrays. In addition, femurs from mice inoculated via intracardiac injection with MDA-MB-231-GFP (parental), MDA-MB-231-PY-GFP (parental), MDA-MB-231-BO-GFP (bone-seeking), or MDA-MB-231-BRMS-GFP (metastasis suppressed) cells or control were assayed ex vivo for cancer cell migration and cytokine production.

MC3T3-E1 murine osteoblasts treated with human metastatic breast cancer conditioned medium produced increased amounts of murine IL-6, VEGF, MIP-2, KC, and MCP-1 with the largest induction seen in 20-day osteoblasts treated with bone-seeking conditioned medium. The human metastatic breast cancer cells themselves produced a similar array of cytokines: IL-6, VEGF, IL-8, and GRO- α with one exception, they produced only small quantities of MCP-1, an important cytokine in angiogenesis, cancer cell survival, and monocyte-osteoclast progenitor cell migration to bone. Indirect transwell co-cultures led to similar results.

The same cytokines were detected ex vivo in femurs of mice bearing human metastatic bone metastases. The metaphyseal ends of long bones, an area of preferential breast cancer cell trafficking, expressed cytokines different from those of the diaphysis. We found that concentrations of IL-6, VEGF, KC, MIP-2, and MCP-1 increased significantly in cancer-bearing mice compared to non-cancer-bearing mice.

Overall, these data suggest that osteoblasts are an important source of cytokines, specifically MCP-1, in breast cancer bone metastasis. These cytokines are known to attract and activate osteoclasts leading to increased bone resorption in bone metastatic breast cancer. Thus, these findings clearly implicate the bone microenvironment and cancer cell manipulation thereof in facilitating metastatic tumor cell colonization and survival.

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P38-14: THE IN VIVO IMPACT OF HOST MMP-2 IN MAMMARY TUMOR-INDUCED OSTEOLYSIS

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Bone metastasis is a common event during breast cancer progression. Growth of metastatic breast tumor cells by inducing the activation of osteoclasts stimulates osteolysis. Osteoclasts are the bone cells responsible for bone degradation and they have been shown to be a rich source of Matrix Metalloproteinases (MMPs). To address the role of stromal MMP-2 to the mammary tumor-growth-induced osteolysis in vivo, we used an

intratibial model that recapitulates breast tumor-induced osteolysis. A mammary tumor cell tagged with luciferase (PyMT-R221A-luc) was injected into FVB wild-type (n=12) and MMP-2 null mice (n=12). Luciferase expression allowed for real-time imaging of the tumors over a period of 9 days. Our results demonstrated that MMP-2 deficient mice showed a significantly reduced tumor growth rate compared to wild-type mice (p<0.04) at both time points. Immunohistochemistry analysis showed that MMP-2 null mice presented a significant higher rate of tumor apoptosis compared to the wild-type mice at day 7 post-injection (p=0.036). However, no difference was observed in tumor proliferation between the two groups of mice. Assessment of bone degradation by staining for mature osteoclasts demonstrated a significant reduction in the number of osteoclasts, in MMP-2 null mice at day 7 (p=0.014) compared to wild-type animals. The volume of bone present in the injected tibia was quantified and we observed a statistical significant decrease in the bone volume in the wild-type mice at day 9 (p=0.01) compared to the MMP-2 null mice. These results suggest that stromal MMP-2 affects tumor-induced osteolysis in vivo by (1) contributing to tumor survival in the bone (2) affecting the number of mature osteoclasts. We are currently investigating the mechanisms through which stromal MMP-2 mediates its effects.

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P38-15: THE ROLE OF HER-2 IN BREAST CANCER BONE METASTASIS

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It has long been recognized that breast cancers have the ability to invade and grow as metastases in the bone. HER2 (ErbB2) is a 185 kDa transmembrane glycoprotein. Approximately 30% of breast cancers overexpress HER2 that initiates aberrant activation of signal transduction pathways causing deregulation of downstream target genes. It has been demonstrated that bone marrow micrometastases express more HER2 than their primary tumor suggesting a selection bias for HER2-overexpressing cells in the bone microenvironment. Here, we sought to understand the role of HER2 in breast cancer bone metastasis thus seeking clinical impact through prognostic tests and preventative therapy, potentially decreasing HER2 mediated bone destruction.

To determine the role of HER2 in breast cancer bone metastasis, we sought to understand the biology of the bone environment when HER2 low- and high-expressing breast cancer cells grow in the bone using an orthotopic model of breast cancer bone metastasis. In our pilot study, we found that when HER2 high- and low-expressing cells were injected into the tibia of nude mice, breast cancer cells that overexpress HER2 created metastases that were slightly more aggressive than that of the parental cell line expressing low levels of HER2. Thus, HER2 may contribute to an increase in osteolytic activity of breast cancer bone metastases. Further experiments are needed to validate the initial findings, which may bring insight on a therapeutic approach for HER2 overexpressing breast cancer bone metastases.

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BREAST CANCER CENTERS OF EXCELLENCE II

Poster Session P39

P39-1: RESEARCH ON OPTIMAL RECOVERY PRACTICES IN BREAST CANCER: THE RESTORE TRIAL

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Background: Post-treatment behavioral interventions, designed to prevent disability and improve quality of life in breast cancer survivors, are needed. Emotional distress, fatigue, and psychosocial issues may be significant and persist over time. The RESTORE study is a randomized, controlled, clinical trial designed to test and compare the effectiveness of a moderate, tailored exercise program on improving quality of life (FACT-B) and physical function (six-minute walk) of women treated for Stage I to III breast cancer at 18-months post-enrollment, versus usual care.

Objectives: Women, with Stage I-III breast cancer treated at one of two large medical centers, were recruited within 6–12 weeks of surgery and randomized to treatment or control. Those in the treatment group began a center-based exercise and a lymphedema education program 2 to 3 days per week gradually shifting to the home. Controls received patient education. Study participants were assessed at 3-month intervals through 18-months. Distributions over time were examined using box plots and smoothed curves. Repeated measures analysis of covariance was used to model the total meters walked over time and FACT-B scores. Models were adjusted for baseline measurement, baseline affected arm volume, number of nodes removed during surgery, age at randomization, number of self-reported symptoms, baseline SF-12 mental and physical components scores, visit and treatment group. Significance was considered to be a 2-sided p-value < 0.05.

Results: There were 105 women in the study, 82 of whom completed all 18 months of the study. Mean age (range) was 53.6 (32–82) years; 88% were Caucasian; 45% were employed full-time; 44% and 27% were overweight and obese. At baseline 46% had breast conserving surgery; 79% had axillary node dissection; 59% received chemotherapy and 64% received radiation. Patients in the treatment group had increased total meters walked compared to controls: adjusted means (SE) were 606.5 (10.7) and 577.1 (6.8) respectively (p=0.025). There was no difference in the adjusted means for FACT-B scores: 117.2 (2.0) for the treatment group and 114.7 (1.5) for the control group (p=0.33).

Conclusions: With this early exercise intervention after breast cancer diagnosis, significant improvement was achieved in physical fitness over time with no decline in health-related quality of life, as measured by FACT-B.

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P39-2: A LONGITUDINAL INVESTIGATION OF POSTTRAUMATIC GROWTH IN WOMEN WITH BREAST CANCER

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Purpose: Posttraumatic growth (PTG) is defined as the experience of “positive psychological change experienced as a result of a struggle with highly challenging life circumstances” (Tedeschi & Calhoun, 2004). In the cancer literature, PTG has been more strongly related to psychosocial variables (optimism, social support, approach-oriented coping, religious coping, psychological well-being) than demographic or medical variables. Yet, the majority of PTG research has been cross-sectional. The current study examines the trajectory of PTG over time and its predictors in women with breast cancer.

Methods: Women recently diagnosed with breast cancer completed surveys at baseline and 6, 12, and 18 months. Surveys included measures of posttraumatic growth (PTGI), coping (COPE), social support (MOS-SSS), spirituality (FACIT-Sp), optimism (LOT), and mental health (SF-36 MCS). Coping was analyzed as a composite score of active-adaptive coping strategies. The PTGI yields a total score and 5 subscale scores: relating to others, new possibilities, personal strength, spiritual change, and appreciation of life. Linear mixed effects models were used to assess the effects of demographic (age, education, race, marital status), medical (stage, time since diagnosis), and psychosocial (social support, spirituality-meaning, spirituality-role of faith, coping, mental health, optimism) variables on PTG.

Results: Participants were 653 women accrued 0–8 months post-breast cancer diagnosis (mean age = 54.9 years, SD = 12.6); most were White (89%) and married/partnered (72%). At study entry, time since diagnosis was a mean of 4.5 (SD = 1.3) months. Cancer stage was mixed: Stage I 52%, Stage II 40%, and Stage III 8%. Percentages of women undergoing the following procedures were: lumpectomy 84%, mastectomy 36%, and reconstructive surgery 20%. They received treatment as follows: radiation therapy 72%, chemotherapy 67%, tamoxifen 41%, and arimidex 33%.

PTG increased over time (p<.0001); compared to baseline, total score increased by 5.2, 6.4, and 8.5 points at 6, 12, and 18 months, respectively. Total PTGI score was most

highly associated with psychosocial variables: greater social support (p=.0019), spirituality-meaning (p<.0001), spirituality-faith (p<.0001), use of active-adaptive coping strategies (p<.0001), and better mental health (p=.0459). Younger age at diagnosis (p<.0001) and non-White racial group (p=.0191) were also associated with higher PTGI. Stage of disease, marital status, and optimism were nonsignificant. Of note, use of active-adaptive coping strategies (e.g., seeking support, positive reframing, planning, and acceptance) was highly associated with PTG; a 1-unit increase in active-adaptive coping predicted an 8.3-point increase on PTGI total score. Additional analyses showed that active-adaptive coping and spirituality variables were consistently associated with PTG for all subscales. Non-white race was only significant for the spiritual change subscale (p<.0001).

Conclusion: Posttraumatic growth increased over time for women with breast cancer, suggesting that many perceive improvements in their lives during and after cancer treatment. Increased social support, spirituality, and use of active-adaptive coping strategies were strongly associated with higher posttraumatic growth in this sample.

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P39-3: SELF-EFFICACY RELATED TO HEALTH-RELATED QUALITY OF LIFE IN BREAST CANCER SURVIVORS

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Purpose: Exercise therapy has been used successfully in the rehabilitation of breast cancer survivors but few intervention studies have examined the link between participants' self-efficacy for exercise and health-related quality of life as recommended by contemporary reviews. Although self-efficacy may play an important role in both physical activity adoption and perceived life quality, this mechanism for behavior change has not been systematically investigated in women treated for breast cancer. Study objectives were to determine intervention effects on self-efficacy and to examine relationships between self-efficacy, health-related quality of life (HRQL), physical activity, and physical function over time.

Sample and Method: Women (n = 104), with Stage I-III breast cancer treated at one of two large medical centers, were recruited within 6–12 weeks of surgery and randomized to treatment or control. Treatment participants began a center-based exercise intervention based on social cognitive theory gradually shifting to the home at 6 mos. Controls received patient education. Demographic characteristics of the sample were: mean age 53.7 years; 88% Caucasian; 45% employed full-time; 43%/28% overweight/obese. At baseline, 46% had breast conserving surgery; 76% had axillary node dissection; 60% received chemotherapy and 64% received radiation.

Results: After intervention, walking self-efficacy (WSE; p<.01) and physical self-efficacy (PSE; p<.05) increased significantly while physical activity participation was significantly greater in intervention versus control at 3 months follow-up (p=.03). After intervention, increased PSE was positively related to increased HRQL (FACT-B) (r=0.57, p<.01) while increased WSE was related to increased total meters walked (r=0.48; p<.01) and increased HRQL (r=0.29, p=.01). An increase in WSE during the first 6 months was correlated with higher frequency of physical activity at 3 months follow-up (r=0.26; p=.03).

Conclusions: This investigation demonstrates that a social cognitive intervention increases self-efficacy and is feasible in this population. These data reinforce the important role of perception when considering the beneficial effects that physical activity has on the health status of women with breast cancer and provide support for the inclusion of physical activity into cancer treatment programs designed to promote function and quality of life. The extent to which changes in social, physical, and behavioral factors may mediate changes in quality of life among breast cancer survivors awaits future examination.

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P39-4: TAILORED COMMUNICATIONS TO ENHANCE ADAPTATION ACROSS THE BREAST CANCER SPECTRUM: CHALLENGES AND INNOVATIVE APPROACHES TO RECRUITMENT AND INTERVENTION DELIVERY WITHIN A BEHAVIORAL CENTER OF EXCELLENCE

Suzanne Miller, Mary Daly, Joanne Buzaglo, Linda Fleisher, Eric Ross, Beth Stearman, Lori Goldstein, Andrea Barsevick, Andrew Balsheim, Carmen Breen-Lopez, and Etyia Faison
Fox Chase Cancer Center

The Behavioral Center of Excellence (BCE) in Breast Cancer was established to provide a comprehensive, multi-disciplinary approach for studying the process of, and

methods for facilitating, successful adaptation in the context of breast cancer risk, treatment, and recovery. The four studies were derived from and integrated by a unifying theoretical framework the Cognitive-Social Health Information Processing model, and were supported by four core facilities (i.e., Administrative, Communication, Genetic Testing and Bioinformatics Core). The four projects were: (1) development of an intervention to promote utilization of breast cancer risk assessment programs and adherence to screening recommendations among underserved African-American women; (2) use of a "teachable moment" and tailored communication materials to promote utilization of risk assessment and adherence to screening among daughters of diagnosed breast cancer patients; (3) the promotion of psychological and physical adaptation among breast cancer patients at the completion of active treatment (i.e., during the re-entry phase); (4) promotion of psychological adaptation among metastatic breast cancer patients. The overarching goal was to develop theoretically guided, tailored, and transportable breast cancer communications to enhance screening adherence, decision-making, and quality of life across the spectrum of disease (i.e., from risk through treatment to survivorship). Challenges in recruitment across these studies included difficulty in accessing and recruiting minority women at familial risk as well as first-degree relatives of breast cancer patients in the community. Further, there were challenges associated with conducting in-person counseling interventions for breast cancer survivors, which led to innovative approaches to intervention delivery including telephone counseling and print materials. Innovative approaches to recruitment and intervention delivery will be discussed with implications for the next generation of tailored interventions for women across the cancer continuum from risk through survivorship.

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P39-5: USING PATIENT SELF-REPORTED DATA TO INCREASE ACCESS AND IMPROVE QUALITY OF CARE

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¹University of California, San Francisco and ²University of California, San Francisco Comprehensive Cancer Center

Background: Informatics systems for breast cancer care are key to improving outcomes in this disease. Prevailing healthcare information systems are generic in their treatment of both the patient and the physician. Only a small fraction of today's systems provide specialized functions supporting oncology care, let alone breast cancer care specifically. In addition, these systems are costly and due to their large size and commercial nature, they evolve too slowly to adapt to the highly dynamic nature of translational science.

We undertook the development of a scalable and extensible healthcare information system designed for breast cancer care. The system requirements involved a platform that was scalable, standards-based, and could be distributed freely without licensing constraints.

Methods: An extensive process was undertaken to evaluate the existing platform. Selection criteria were developed and consisted of a matrix of items. The criteria included: (1) web-enabled, (2) support for healthcare data and coding standards, (3) support for caBIG standards, (4) support for the HL-7 reference information system (RIM) standard, (5) extensibility, (6) and availability for re-distribution without licensing constraints.

Results: A search for platforms that adhered to the criteria was undertaken. A small set of platforms was found to conform to the criteria. These platforms were further explored through the development of prototype applications. Ultimately, we selected a recently available open-source platform designed for healthcare applications (Tolven). The system is web-based and built on an open source technology stack (jBoss, PostgreSQL). The system is healthcare centric and based on the HL-7 RIM. The platform supports the use of standard terminologies for representation of data as well as caBIG data standards. We found the system also incorporated innovative approaches to security including the real-time encryption/decryption within the database in real-time, a method we have not seen in other systems. The system information model supports both patient and physician views of the same data, but with appropriate constraints on data access, modification, and ownership. The open source nature of the platform met the criteria for free re-distribution.

Conclusions: An open source platform, web-enabled, standards based platform exists for the development of healthcare applications. This allows the development of customized breast cancer care systems which can integrate with prevailing electronic healthcare systems through HL-7 interfaces. The support of standard terminology systems allows for a degree of semantic interoperability between these systems as well. Future research will involve characterizing the degree to which interoperability between the breast cancer system built with Tolven can achieve with an institutional electronic medical record system as well as caBIG derived systems such as caTISSUE. This

research will provide insights in how best to develop specialty-specific information systems that can interoperate with generic electronic healthcare record systems being implemented in most healthcare organizations today.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0481.

P39-6: THE COORDINATED DIAGNOSTIC AND EVALUATION PROGRAM: AN INNOVATIVE APPROACH TO DELIVERING BETTER CARE TO WOMEN WITH SUSPICIOUS BREAST IMAGING

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Background: Women who receive a call back after screening mammography often wait weeks to months to get resolution and, once a diagnosis of cancer is received, they still have to make appointments to discuss care plans with a surgeon. BIRADs 4 mammograms are called suspicious and include a range risk of DCIS or cancer from 3%–75%. Diagnostic delay, unnecessary tests, and prolonged psychological distress place additional burdens on patients (pts). There is a very significant problem given that 6%–10% or more of women who obtain screening mammograms are called back, which may affect up to 5 million women in the U.S. per year.

Objectives: In fall of 2005, we established the Coordinated Diagnostic Evaluation Program (CDEP) - a one-stop multidisciplinary clinic which provides comprehensive diagnostic services to women with suspicious mammograms (BIRADs 4, 5). We hypothesized that the CDEP would improve the care experience by shortening diagnostic work-up time, relieving excessive anxiety from waiting, identifying low risk pts where procedures can be minimized, instantly coordinating next steps of care for women with cancer, and identifying high risk patients who may benefit from preventative interventions.

Methods: The complete clinical, pathological and follow-up data of all pts seen at the CDEP clinic between January 2006 and March 2007 has been prospectively collected. We analyzed 216 pts out of 230 seen in the clinic. Suspicious imaging (BIRADs 4 and 5) was the main criteria for enrollment. We collected key variables: age, Gail risk score, breast density, BIRADs score, risk estimate (RE) of invasive cancer and DCIS based on the prior-to-CDEP imaging, number and type of procedures and imaging performed in the CDEP work-up, and finally the time to and the type of final resolution.

Results: Patients with BIRADs 0, 1 and 2, 3, 4, 5 constituted 15%, 3%, 8%, 53%, and 6% of CDEP patients respectively. In addition, 14% of pts had breast symptoms alone. The mean age was 52 years. Risk estimate (RE) for DCIS or invasive cancer was obtained prospectively. A total of 79 pts had RE greater than 20%, of which 42% (32 pts) had a diagnosis of DCIS or invasive cancer. Eighty-one percent of cancers had BIRADs of 4 and 5 and 19% had BIRADs of 0. For pts with RE less than 20%, none had cancer. Final resolution was achieved on the CDEP day (71%), 2 weeks (2.8%), one month (9.3%), or more (14.3%). A total of 75% of women (15 out of 20) with the final resolution time of one month required excisional biopsy. Biopsies were avoided in 42% of pts.

Conclusions: The CDEP process streamlined evaluation into an effective, patient-centered, multidisciplinary clinic day, successfully coordinating care. Time to resolution was dramatically improved for women in the CDEP clinic with 71% getting diagnosis on the CDEP day. To maximize use of resources and minimize anxiety, women with at least 20% chance of malignancy (BIRADs 4b, 4c) should be referred to CDEP. Women with less than 20% chance of malignancy (BIRADs 4a) should be told that their risk is minimal, and we should pilot less invasive methods for evaluation. Finally, a program that provides outcome measures as a byproduct of care enables continuous improvement in service.

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P39-7: DEVELOPMENT OF AN INTEGRATED INFORMATICS PLATFORM FOR BREAST CANCER CARE

Laura Esserman and Michael Hogarth
University of California, San Francisco

Background: Informatics systems for breast cancer care are key to improving outcomes in this disease. Prevailing healthcare information systems are generic in their treatment of both the patient and the physician. Only a small fraction of today's systems provide specialized functions supporting oncology care, let alone breast cancer care specifically. In addition, these systems are costly and due to their large size and commercial nature, they evolve too slowly to adapt to the highly dynamic nature of translational science.

We undertook the development of a scalable and extensible healthcare information system designed for breast cancer care. The system requirements involved a platform that was scalable, standards-based, and could be distributed freely without licensing constraints.

Methods: An extensive process of evaluation was undertaken to evaluate existing platform. Selection criteria were developed and consisted of a matrix of items. The criteria included: (1) web-enabled, (2) support for healthcare data and coding standards, (3) support for caBIG standards, (4) support for the HL-7 reference information system (RIM) standard, (5) extensibility, (6) and availability for re-distribution without licensing constraints.

Results: A search for platforms that adhered to the criteria was undertaken. A small set of platforms was found to conform to the criteria. These platforms were further explored through the development of prototype applications. Ultimately, we selected a recently available open-source platform designed for healthcare applications (Tolven). The system is web-based and built on an open source technology stack (jBoss, PostgreSQL). The system is healthcare centric and based on the HL-7 RIM. The platform supports the use of standard terminologies for representation of data as well as caBIG data standards. We found the system also incorporated innovative approaches to security including the real-time encryption/decryption within the database in real-time, a method we have not seen in other systems. The system information model supports both patient and physician views of the same data, but with appropriate constraints on data access, modification, and ownership. The open source nature of the platform met the criteria for free re-distribution.

Conclusions: An open source platform, web-enabled, standards based platform exists for the development of healthcare applications. This allows the development of customized breast cancer care systems which can integrate with prevailing electronic healthcare systems through HL-7 interfaces. The support of standard terminology systems allows for a degree of semantic interoperability between these systems as well. Future research will involve characterizing the degree to which interoperability between the breast cancer system built with Tolven can achieve with an institutional electronic medical record system as well as caBIG derived systems such as caTISSE. This research will provide insights in how best to develop specialty-specific information systems that can interoperate with generic electronic healthcare record systems being implemented in most healthcare organizations today.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0481.

P39-8: DECISION SERVICES AT THE UCSF BREAST CARE CENTER

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Background and Objectives: We established Decision Services in 2003 as part of the Breast Care Center (BCC) at the University of California, San Francisco (UCSF), with funding from a Department of Defense (DOD) grant (DAMD17-03-1-0481). Our objective is to reach all UCSF patients, caregivers, students, and doctors who desire assistance with or training in decision making. Our larger purpose is to improve the quality of decision making in health care.

Methods: Decision Services engages in patient care, research, teaching, and community service. Patient care includes patient education; consultation planning, recording, and summarizing; and decision aiding. Patient education involves sending patients educational videos and booklets produced by the Foundation for Informed Medical Decision Making. Our consultation planning, recording, and summarizing program employs premedical interns to accompany patients to their appointments and help them list questions, take notes, and audio-record the consultation with the doctor. Our decision aiding program allows doctors to guide patients to good decisions based on presentations of individualized risks and benefits.

Our research program focuses on creating, implementing, and evaluating decision support interventions at UCSF; with diverse populations in rural, underserved areas of Northern California; and with other populations through national and international collaborations.

As part of our teaching program, we offer an elective on decision making to first-year medical students; a lecture on shared decision making in the "Cancer Block" to second-years; and a lecture on clinical decision making to third-years. We also offer continuing medical education workshops on the topic of Guiding Patients to Good Decisions and Promoting Patient Participation in Medical Care. We provide, as a community service, workshops for patients, survivors, and the general public on the topic of Making Good Decisions about a Health Crisis in Your Family. We host a website for the general public at www.guidesmith.org

Results: In 2007, we sent 426 videos to 332 newly diagnosed patients, and created 237 consultation plans, recordings, and summaries for patients visiting our surgeons and oncologists. Our programs were associated with significant improvements in patient decision self-efficacy (+9%), knowledge (+33%), and decisional conflict (-20%). We had 8 active research protocols. We conducted 10 educational programs for more than

461 doctors and students, and put on 2 workshops for more than 46 members of the general public. The guidesmith.org website had 8,151 unique visitors in 2007 with 21,367 page views.

Conclusion: With Decision Services, we have successfully integrated decision support into the routine workflows of the BCC. We are disseminating our findings and other best practices by training premedical interns, medical students, doctors, and the general public. The Decision Services unit contributes to the BCC's overall vision of tailoring care to patient preferences and biology, based on medical evidence and clinical performance. Our current goals are technological and organizational. Regarding technology, we seek to streamline and automate the provision of decision support through better software tools. Organizationally, we seek better integration of Decision Services with surgeons, oncologists, and other providers who serve as trusted guides for patients navigating complex treatment decisions.

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P39-9: DEVELOPMENT OF A COMPUTERIZED DECISION AID FOR BREAST CANCER PREVENTION

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Background: We are developing a computerized decision aid for breast cancer prevention. The decision aid is designed for use during consultations between provider and patient and uses risk stratification and patient preference to tailor breast cancer prevention interventions.

Methods: The decision aid provides a framework for breast cancer risk management using a shared decision-making process and is based on a decision algorithm that includes a general health and breast cancer risk assessment in the context of age-matched women and evidence-based models using biomarkers (atypia, BRCA1/2 mutations) as risk discriminators and predictors of benefit from possible interventions.

Results: Feasibility of the decision aid was explored in a clinical trial at the UCSF Breast Cancer Center. Eligible, consented women were randomized to either a control group using the standard format for their prevention consultation, or an intervention group using the computerized decision aid during their prevention consultation. Based on the pilot trial results, the decision aid was found to be feasible; the decision aid did not significantly increase the duration of the consultations and all patients using the decision aid found the intervention acceptable. Physicians' ease of use and comfort with the decision aid increased as the trial progressed. Patients generally found the decision aid informative and non-intrusive. Patients were interested in biological markers that refine risk and benefit of intervention and this risk information had an impact on patient decision-making.

Conclusions: The decision aid provides access to key information during consultations and allows the integration of emerging biomarkers in the prevention setting. Initial results suggest that the decision aid is clinically feasible and patients found that a better understanding of their risk was useful in decision-making. Future applications of the decision aid include a large randomized trial to determine impact on decision-making, web-based access, and the integration of tools to store data and track decisions and outcomes.

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P39-10: PREGNANCY FOLLOWING BREAST CANCER TREATMENT: A PROSPECTIVE COHORT ANALYSIS

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Introduction: Even though apparently normal menstrual cycling may be maintained after chemotherapy for breast cancer, the incidence of pregnancy and successful child-bearing is unknown, largely due to a lack of prospective cohort studies in this area. This paper reports pregnancy data from participants enrolled in a prospective, longitudinal study of premenopausal breast cancer patients.

Methods: Between 1998 and 2005, 836 women, ages 20-45 years, diagnosed with Stage I-III breast cancer, were recruited to the Menstrual Cycle Maintenance and Quality of Life After Breast Cancer Study. All women had regular menstrual cycles at the time of diagnosis. Study follow-up continues. The average age at recruitment was 39 years (i.e., 25% < age 35, 30% ages 35-39, and 45% age 40+). About 88% of the participants are non-Hispanic White, 68% have at least a 4 year college degree, and 75% are married or partnered. Most participants were diagnosed at stage 1 (41%) or stage 2 (52%). Sixty-four percent had estrogen receptor positive (ER+) disease. 51% had a mastectomy, and 88% received chemotherapy, 69% received radiation, and 58% received hormone therapy.

Results: At the time of recruitment, 70% of the participants had had at least 1 full-term birth; 75% had been pregnant. When asked about plans for future childbearing, 19% (160) desired a future pregnancy, and 15% (125) were undecided. As of December 1, 2007, 103 pregnancies among 70 women had been reported. The time to first pregnancy was approximately 32 months (SD=18.8) after diagnosis or 28.5 months (SD=18.6) after the end of chemotherapy. The mean number of pregnancies per woman was 1.5 (SD=0.76), with no woman reporting more than 4 pregnancies. Of these 103 pregnancies, 70 were live births (including 4 sets of twins), 1 was a surrogate birth (a set of twins), 2 were live births as a result of donor eggs, 24 were miscarriages, and 6 were terminations. Since these pregnancies/births, 3 participants and 1 infant have died. The majority (77%) of the pregnancies did not necessitate the use of assisted reproductive techniques. However, the techniques reported by participants in one or multiple pregnancies were: in-vitro fertilization (4), donor eggs (4), own eggs (2), surrogate mother (1), artificial insemination (2), clomid (2), hysterosalpingogram (1), and fertility acupuncture treatments (1).

In comparing characteristics of the 70 women who became pregnant with the total sample of participants, the 70 women were younger at recruitment (mean age 33.6 [SD=3.8] years), were diagnosed at slightly earlier stages of disease (51.5% at stage 1; 47% at stage 2), and had a lower proportion with ER+ disease (53%). 93% had chemotherapy, and 74% received radiation. We also collected information on participants who were unsuccessful in their pregnancy attempts. To date, only 19 women have reported being unable to conceive. The major distinguishing characteristic between these women and those who have conceived is age. On average, these 19 women were 38.2 years (SD=4.3) at recruitment, and were in their late 30's and early 40's when attempting to become pregnant.

Conclusion: We will continue to collect pregnancy data from this cohort prospectively. The tracking and reporting of these data are critical to increasing our knowledge of pregnancy and outcomes following breast cancer treatment.

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P39-11: MENSTRUAL BLEEDING AFTER LONG-TERM AMENORRHEA FOLLOWING BREAST CANCER TREATMENT: RESULTS BY AGE

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Introduction: Previous research indicates that chemotherapy improves survival especially in premenopausal women. However, improvements in survival rates can lead to chemotherapy-induced amenorrhea (CIA) among younger patients. CIA is often associated with premature ovarian failure with resultant infertility and symptoms such as hot flashes, mood changes, atrophic vaginitis, and dyspareunia. Other significant long-term effects include osteoporosis, increased risk of fractures and cardiovascular disease. On the other hand, CIA may also benefit patients in increasing rates of disease free survival. We present the results of a prospective, multicenter study designed to examine the effects of chemotherapy on amenorrhea and the resumption of menstrual bleeding in premenopausal women. Specifically, we examine the incidence of CIA, as well as the resumption of bleeding, by age, following 6, 12, and 24 months of amenorrhea post-chemotherapy.

Methods: Between January 1998 and July 2002, 491 women ages 20-45 years old with regular menstrual cycle and stage I-III breast cancer were recruited to the Menstrual Cycle Maintenance and Quality of Life After Treatment Study. Patients were followed prospectively and completed monthly bleeding calendars from the time of recruitment. Updated medical history data were obtained at 6 month intervals thereafter with a maximum follow-up of 6 years.

Results: The majority of participants received AC (doxorubicin, cyclophosphamide), ACT (doxorubicin, cyclophosphamide, paclitaxel) or CMF (cyclophosphamide, methotrexate, and 5-fluorouracil). Of 491 women, 439, 445, and 403 patients returned more than 50% of their bleeding diaries and were included in the analysis of 6, 12, and 24 months of CIA, respectively. Rates of CIA across all age groups were 41%, 29%, and 23% for 6, 12, and 24 months of CIA, respectively. Resumption of bleeding within 3 years of the amenorrheic period occurred in 48%, 29%, and 10% of women undergoing 6, 12, and 24 months of CIA. As expected, rates of amenorrhea and resumption varied by age. For patients ages 20-34 years, rates of 6, 12 and 24 months of CIA were 11%, 5%, and 1%, respectively. Resumption of bleeding within 3 years of the amenorrheic episode occurred in 79%, 50%, and 0% of women undergoing 6, 12, and 24 months of CIA. For patients ages 35-39, rates of 6, 12, and 24 months of CIA were 28%, 16%, and 11%, respectively. Resumption of bleeding within 3 years occurred in 66%, 43%, and 0% of women undergoing 6, 12, and 24 months of CIA. For women over 40 years of age, rates of 6, 12, and 24 months of CIA were 65%, 49%, and 40%, respectively. Resumption of bleeding within 3 years of the amenorrheic episode occurred in 41%, 25%, and 12% of women undergoing 6, 12, and 24 months of CIA.

Conclusion: Permanent CIA in women younger than 40 years old is low, with most resuming cycling within 6-12 months after the end of chemotherapy. Rates of CIA in women 40 years and older are higher and less likely to be followed by a resumption of bleeding. However, patients greater than 40 years old, even after 2 years of CIA, can have a return of menstrual bleeding. Therefore, biochemical testing should be obtained if there is any consideration for switching to an aromatase inhibitor.

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P39-12: RACIAL DIFFERENCES IN THE INTERACTION BETWEEN DNA METHYLATION PHENOTYPES AND ONE CARBON METABOLISM GENETIC VARIANTS IN NORMAL BREAST TISSUES

Ramona Gianina Dumitrescu,¹ Catalin Marian,² Shiva Krishnan,² Bhaskar Kallakury,² Francoise Seillier-Moiseiwitsch,² Habtom Ressim,² Scott Spear,² Jo Freudenheim,³ and Peter G. Shields²

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DNA hypermethylation of tumor suppressor genes promoter region and global DNA hypomethylation are thought to be common and early epigenetic events in breast carcinogenesis. In this study, we examined potential determinants of DNA hyper- and hypomethylation in normal breast tissue, including genetic variants for genes important to one-carbon metabolism.

We recruited 141 women without a history of breast or other cancers, who were undergoing reduction mammoplasty. These women underwent a structured interview and donated breast tissue and blood. Hypermethylation phenotypes for *p16 INK4*, *BRCA1*, *ERα*, and *RAR-β* from the normal breast tissues were determined by MSP and Pyrosequencing assays. Global DNA methylation was assessed by LINE-1 Pyrosequencing. Genetic variants of *MTHFR* and *MTR* genes were determined by TaqMan assays. Chi-square, t-tests, and logistic regression were used to determine the association between promoter hypermethylation of these genes and genotypes and other characteristics of these women. Comparisons for global hypomethylation were made with non-parametric one-way analyses of variance by ranks.

We observed that *p16 INK4*, *BRCA1*, *ERα*, and *RAR-β* promoter hypermethylation is present in 31%, 16%, 9%, and 0% of the normal breast tissues from healthy women undergoing reduction mammoplasty. The global methylation level range was 65.34-82.78%, with mean 73.87%, median 73.30%, and SD 4.60%. Race was significantly associated with the methylation status and when we stratified these women by race we observed that family history of cancer, the variant allele of the *MTR A2756G* polymorphism and alcohol consumption were associated with *p16 INK4* hypermethylation only in Caucasian women. There were no significant associations for African American women. *BRCA1* hypermethylation was significantly associated with family history of cancer for African American women. Family history of cancer in Caucasians and family history of breast cancer for African American women were weakly associated with *BRCA1* methylation. *ERα* methylation phenotype was significantly correlated with the age of both Caucasian and African American women. Moreover, family history of breast cancer or any other cancer and the variant allele *MTR 2756G* were also weakly associated with *ERα* hypermethylation in African American women. For global hypomethylation there were significant associations with family history of cancer and with variant alleles in *MTR 2756G* and *MTHFR 1298C*, and a marginal association with current smoking status only for Caucasians.

There were important differences in these associations by race. It was observed that DNA hypermethylation at the promoter region of important tumor suppressor genes is a common finding in apparently healthy women without history of breast cancer. Several known risk factors for breast cancer were associated with increased risk of hypermethylation and global hypomethylation, as were genetic variants for *MTR* and *MTHFR* gene. Understanding the determinants of hypermethylation and global hypomethylation in normal breast tissues for different races can provide insight into a potentially significant mechanism for breast carcinogenesis.

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P39-13: ALCOHOL CONSUMPTION AND DNA HYPERMETHYLATION IN BREAST CANCER: THE WESTERN NEW YORK EXPOSURES AND BREAST CANCER (WEB) STUDY

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Epidemiological studies have shown a modestly increased risk of breast cancer with alcohol consumption in both pre- and postmenopausal women. One possible mecha-

nism for this association may be the effect of alcohol on one-carbon metabolism possibly leading to an imbalance in DNA methylation. As an epigenetic modification, DNA promoter hypermethylation has been implicated in the pathogenesis of solid tumors, including breast cancer. However, frequency of hypermethylation in breast cancer in relation to alcohol consumption has not been well studied. Using archived tumor blocks from incident breast cancer cases in a population-based Western New York Exposures and Breast Cancer Case Control (the WEB) Study conducted in western New York between 1996 and 2001, we evaluated the likelihood of promoter hypermethylation of genes in breast tumors, including *E-cadherin*, *p16*, and retinoic acid β_2 receptor (*RAR- β_2*), in association with lifetime alcohol consumption.

DNA samples isolated from 803 paraffin-embedded tumor tissue were analyzed for hypermethylation status using real-time methylation-specific polymerase chain reaction (MSP). Alcohol consumption was queried by interview regarding lifetime consumption. Case-case comparisons of hypermethylated and unhypermethylated cases to determine the relative prevalence of hypermethylation of each gene by exposure to lifetime alcohol consumption were conducted using unconditional logistic regression and 95% confidence intervals (CI) were calculated.

There were 161, 208, and 221 cases with hypermethylation of *E-cadherin*, *p16*, and *RAR- β_2* gene, respectively. For postmenopausal breast cancer, comparing to never drinkers, there was increased likelihood of *E-cadherin* gene hypermethylation in tumors from lifetime ever drinkers (OR = 2.21, 95% CI, 1.10–4.45). Similarly, there were increased likelihoods of tumors with *E-cadherin* gene hypermethylation, for examination of alcohol consumption 2, 10 to 2, and 20 to 10 years prior to cancer diagnosis. The increased likelihood of *E-cadherin* gene hypermethylation for ever drinkers compared to never drinkers was particularly stronger for estrogen receptor (ER) negative tumors (OR = 4.31, 95% CI, 1.19–15.58).

Conversely, there was decreased likelihood hypermethylation of *p16* gene among lifetime ever drinkers compared to never drinkers (OR = 0.53, 95% CI, 0.31–0.91). *RAR- β_2* hypermethylation was not associated with alcohol consumption. We did not observe any association of alcohol consumption and promoter hypermethylation among premenopausal breast cancer for any of the three genes. There were no differences in hypermethylation of the three genes by folate intake.

In summary, we found evidence that alcohol consumption may be associated with increased hypermethylation of *E-cadherin* and decreased hypermethylation of *p16* gene in postmenopausal breast cancers. Changes in promoter methylation may explain part of the observed association of alcohol with breast cancer risk.

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P39-14: ADH1B AND ADH1C HAPLOTYPE TAG SNPS: ASSOCIATIONS WITH BREAST CANCER RISK AND INTERACTIONS WITH ALCOHOL CONSUMPTION IN THE WESTERN NEW YORK EXPOSURES AND BREAST CANCER (WEB) STUDY

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One of the most consistently documented risk factors for breast cancer, other than familial and hormonal risk factors, is alcohol drinking. Few data exist to date on the association between polymorphisms in alcohol dehydrogenase genes and breast cancer risk. Here we investigated the effect of haplotype tag SNPs in *ADH1B* and *ADH1C* on breast cancer risk and the interaction with alcohol drinking in a population-based case control study (the WEB Study) conducted in western New York between 1996 and 2001. Genomic DNA was extracted from blood or buccal cells of cases and controls and SNP genotyping was performed by allelic discrimination real-time PCR with Taqman probes. Haplotype tag SNPs were selected from the Caucasian HapMap data. Alcohol consumption was queried by interview regarding lifetime consumption. Logistic regression models were used and backwards selection was employed to obtain the most parsimonious model starting with the full model with interactions, and 95% confidence intervals (CI) were calculated. Two SNPs, rs1042026 in *ADH1B* and rs1614972 in *ADH1C*, were associated with risk of postmenopausal breast cancer. rs1042026, while not in a functional region, has been shown to be in linkage disequilibrium with several SNPs that are functional or could be functional from *ADH6*, *ADH1A*, *ADH1B*, and *ADH1C*. Importantly this SNP was not in Hardy Weinberg equilibrium in the postmenopausal cases, only in postmenopausal controls, strengthening evidence of association. There was a significant association with risk for interaction of the rs1042026 genotype and alcohol consumption during the period 2–10 years previous, with presence of the C allele (dominant coding) increasing the odds of postmenopausal breast cancer in women who consume alcohol versus those who abstain by almost

2-fold (OR=1.9453). In addition to finding a significant difference in odds of breast cancer in drinker versus abstainer when considering consumption across three levels, we found that the presence of the C allele increased risk for low and high levels of drinking (p=0.0029) and that this effect approached differing significantly depending on amount of alcohol consumption (p=0.08). The second SNP, rs1614972, located in intron 8 of *ADH1C*, is in LD with other six nonsynonymous exonic SNPs including the functional 1350V (rs698). There was evidence of an interaction of the CC genotype with alcohol consumption in association with risk; CC was associated with a protective effect varying inversely with the drinking level (p=0.0092). Moreover, the CC genotype decreased the odds of breast cancer in women who consume alcohol versus those who abstain regardless of menopausal status (OR=0.56, 95% CI 0.35-0.90). In conclusion, haplotype tag SNPs in *ADH1B* and *ADH1C* were associated with breast cancer risk especially in postmenopausal women, and showed evidence of significant interaction with alcohol consumption.

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P39-15: GENETIC VARIATION IN ONE CARBON METABOLISM ENZYMES AND RISK OF BREAST CANCER: THE WESTERN NEW YORK EXPOSURES AND BREAST CANCER STUDY (WEB STUDY)

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The one-carbon metabolism pathway is of potential interest in understanding breast carcinogenesis. There are several enzymes involved in one carbon metabolism whose genes are polymorphic and may relate to breast cancer risk. The *MTHFR* gene encodes for the enzyme methylenetetrahydrofolate reductase and is responsible for the irreversible reduction of the 5, 10 methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reduction provides methyl groups for the remethylation of homocysteine to methionine required for the production of S-Adenosylmethionine (SAM) and tetrahydrofolate (THF). The *MTR* gene encodes for the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase, also referred to as the methionine synthase gene which catalyzes the remethylation of homocysteine. SAM is required for methylation reactions and THF, the fundamental folate acceptor molecule for one carbon metabolism is used in nucleotide synthesis.

There are two common variants of the *MTHFR* gene (C677T, rs 180113 and A1298C, rs 1801131) and a common variant of the *MTR* gene (A2756G, rs1805087). We investigated the main effect of these polymorphisms as well as interactions with alcohol intake and with each other in a population-based case control study conducted in two counties of western New York. Cases (n=1170) were women with primary incident pathologically confirmed breast cancer and controls (n=2115) were frequency matched to cases on age and race. Genotyping (1049 cases and 1875 controls) was done by real-time PCR allelic discrimination with TaqMan probes and lifetime alcohol consumption information was collected by trained interviewers. Odds ratios (OR) and 95% confidence intervals (CI) were estimated by unconditional logistic regression adjusted for age, education and breast cancer risk factors and stratified by menopausal status.

We found no associations of *MTHFR* or *MTR* genotypes with risk. The adjusted ORs for variant genotypes of the *MTHFR* and *MTR* genes for pre- and postmenopausal breast cancer were as follows: *MTHFR* C677T CT and TT: 0.93 (0.69-1.27), 1.10 (0.91-1.33), *MTHFR* A1298C AC and CC: 0.97 (0.71-1.33), 0.94 (0.78-1.14), *MS* A2756G AG and GG: 0.81 (0.59-1.11), 1.02 (0.85-1.24), respectively. Among postmenopausal women with the TT variant genotype for *MTHFR* C677T, there was a statistically significant increase in risk (OR=1.71, 1.01-2.90) for those whose lifetime alcohol intake was above the median intake compared to nondrinkers with the common CC genotype. There were no associations of the A1298C *MTHFR* genotype or the *MTR* genotype with risk within categories of alcohol intake. We found evidence of interaction of *MTHFR* and *MTR* genotypes; compared to those who were homozygous wild type for all genotypes, there was increased risk for those who had more than one variant genotype (OR=2.14 (0.78-5.87)); however, cell sizes were small and the confidence interval wide.

In this case control study of *MTHFR* and *MTR* genotype and risk of breast cancer, we found no evidence of risk associated with main effects of genotype, but found evidence for an increase in risk among postmenopausal women with the variant for C677T *MTHFR* who had higher lifetime alcohol consumption and a suggestion that having more than one variant genotype for this combination of genes might increase risk.

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P39-16: SPECTRUM OF mtDNA SOMATIC MUTATIONS IN BREAST CANCER AND THEIR POTENTIAL SIGNIFICANCE EVALUATION

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Mitochondria are indispensable in energy metabolism, apoptosis, oxidative stress, and aging, and so a role for mitochondria in cancer causation has been implicated. Mutations in the mitochondrial DNA (mtDNA) are common in cancer. In this study, we determined the mtDNA mutational spectrum in 128 breast cancer patients by PCR, TTGE mutation screening, and sequencing identification to investigate spectrum and frequency of mtDNA somatic mutations in breast cancer and to relate them to clinical factors and epidemiological risk factors. It was found that 60 tumors had somatic mtDNA mutations compared to the control germline DNA, with a range of 1–5 mutations. Of the 60, twenty-nine tumors had single mutations (48.3%). Each of the remaining 31 tumors (51.7%) had multiple somatic mutations. Forty-three (33.6%) tumors had mutations in the hypervariable D-loop region and 14 tumors (10.9%) had mutations in the mRNA region. Of the 119 somatic mutations observed, 12 (10.1%) are short deletions or insertions, and the remaining 107 (89.9%) are point mutations. Fifteen were nonsynonymous mutations in coding region, including T4112A(L269Q), C4216T(H304Y), C4248T(T314I), A4527C(T20P), G4769A(V100M), A11567G (I270V) in NADH dehydrogenase subunit; C8448T(T28M), G9182C(S219T) in ATP synthase F0 subunit; and G9621A(A139T), G9985A(G260E) in cytochrome c oxidase subunit gene, etc. Thirteen mutations were at synonymous coding region or at ribosomal and transfer RNAs, and 43 involved noncoding segments (D-loop). When the number of somatic mutations was normalized to the size of the region, it was found that the D-loop region was more than 40 times more susceptible to mutation than the coding region (90 mutations in 1,122 bp of D-loop region versus 29 mutations in 15,358 bp of coding regions, the average mutation densities are 6.22 and 0.14/1000bp/person, respectively). We also found an insertion or deletion at the np303–309 poly C tract region of D-loop region in 9 (7%) tumors compared with 21%–72% in previous reports. This study revealed a high frequency and a variety of somatic mtDNA mutation exists in breast cancer. Although some of these mutations are located at a structurally/functionally important region and may affect the biogenesis of mitochondria, there is no single mutational hotspot associated with the wide spectrum of breast cancer patients.

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P39-17: CLINICAL AND PATHOLOGIC CORRELATES IN BREAST CANCER BRAIN METASTASES: TISSUE MICROARRAY STUDIES

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Background: Brain metastasis (BM) occurs in approximately 30% of breast cancer (BC) patients. Molecular mechanisms of central nervous system (CNS) tropism and dissemination are not well understood. This study aimed to correlate tumor signatures with clinical variables and compare pathologic characteristics of matched BC primary and BM tissue.

Materials and Methods: Clinical data extracted from medical records of 209 BC patients (pts) with available BM tissue treated at Memorial-Sloan Kettering Cancer Center from 1989 to 2006 were included in the retrospective analysis. Of 56 primary BC and 133 BM formalin-fixed, paraffin-embedded tissue samples available, 43 were paired, and all were analyzed using TMA. Standard BC pathologic features such as ER, PR, and HER2 as well as EGFR and c-kit were evaluated. We also assessed cytokeratins (CK) 5/6, 4A4 and calponin, markers associated with basal-like breast cancers. Clinical variables were compared with pathologic markers using Fisher's exact, Wilcoxon, or Kruskal-Wallis tests. Differences in survival times from diagnosis of BM to death due to disease or last follow-up were tested by log-rank. McNemar's exact test was used to assess differences in paired proportions of matched TMA samples. For all tests, $p < .05$ was statistically significant.

Results: The median age at BC diagnosis was 45 years (range, 26–83) and at BM, 50 years (range, 30–83). The median time from BC diagnosis to BM was 37 months (range, 0–432). Neither age at BC diagnosis (≤ 45 versus > 45 years) nor race (white versus black versus other) was associated with interval from BC diagnosis to BM. Median survival from diagnosis of BM was 19 months, 95%CI (13, 24); 27 months for pts with solitary BM and 11 months for pts with multiple BM ($p < 0.0001$). There was no survival difference by first site of metastasis (CNS versus any other). Node-negative pts have a longer time interval from BC to BM as compared to node-positive pts (43 versus 34 months, $p = 0.02$). Smaller (T1) breast cancers metastasized to brain after a median of 41 months, while larger (T2 and T3) tumors metastasized after 27 and 30 months, respectively ($p = 0.03$); this relationship was also noted in an analysis restricted to node-positive pts (T1: 39 mos. versus T2-3: 25 mos., $p = 0.04$). Among 133 BM, no differences in brain TMA markers were observed between pts with solitary

versus multiple BM. Among the 56 primary BC TMAs, there was a marginally higher proportion of multiple BM in pts with CK 5/6 overexpressing primary breast cancers ($p = 0.07$). Comparison of paired breast primary and BM TMAs revealed no significant discordance among markers ER, PR, HER2, EGFR, or CK 5/6.

Conclusions: Smaller tumor burden (T-size and nodal status) at initial BC diagnosis was associated with a significantly longer time interval before brain metastasis. Paired analysis of molecular profiles for matched primary BC and metachronous BM tissue showed no significant discordance between the assessed pathologic features. No TMA signature distinguished pts more likely to develop multiple versus solitary BM, though CK 5/6 expression in the breast primary tumor may be possibly associated with greater likelihood of multiple BM. Further analysis of potentially relevant markers will be directed by ongoing animal models of BC BM.

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P39-18: GENE EXPRESSION ANALYSIS FOR PREDICTION OF EARLY BRAIN METASTASIS (BM) IN HER-2 POSITIVE BREAST CANCER PATIENTS (PTS)

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Background: BM is a common occurrence in HER2+ breast cancer pts. If the pts most likely to develop BM could be identified, prophylactic strategies might prevent or delay occurrence of this failure. We explored gene expression differences between HER2+ breast cancers with early versus late occurrence of BM.

Methods: Study group included 90 HER2+ breast cancer pts, 43 of whom developed BM (BM+); 22 and 21 pts with ≤ 3 and > 3 yrs in time from diagnosis to BM, respectively; 47 patients had not developed BM (BM-) at the last follow-up. We performed cDNA-mediated annealing, selection, extension, and ligation (DASL) assay (Illumina Corp) for expression of 502 known cancer genes using 200 ng RNA from archived FFPE. T-test with unequal variances was applied after sample median normalization. Differentially expressed genes were analyzed using ingenuity pathway analysis.

Results: A binary comparison of BM+ versus BM- revealed 25 differentially expressed genes (p -value < 0.05). For BM+ in < 3 yrs versus > 3 yrs comparison, 95 genes were differentially expressed with a p -value < 0.05 . Upregulated gene pathways included glucocorticoid receptor, PI3K/AKT and PTEN, IGF-1, P53, and NF- κ B. Downregulated gene pathways were cell cycle G1/S checkpoint regulation, cell cycle G2/M DNA damage checkpoint, vitamin D, and retinoic acid receptor signaling.

Conclusions: Early BM occurrence in HER2+ breast cancer pts can be predicted by gene expression in primary tumors. Altered cell cycle regulation seems to be particularly important. Analyses are ongoing to generate a gene expression signature to predict development of BM.

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P39-19: ANTICANCER DRUG UPTAKE AND DISTRIBUTION IN MDA-MB-231BR BRAIN METASTASES OF BREAST CANCER

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Introduction: Chemotherapy has shown limited efficacy in the treatment of brain metastases of breast cancer. One factor implicated in this failure is restricted drug penetration across tumor vasculature. Normal brain is protected from many circulating xenobiotics and toxins by a low vascular permeability coupled with high expression of efflux pumps. We have shown in an accompanying abstract that the integrity of this barrier is compromised in a novel mouse model of human brain metastases of breast cancer. In this study, we examined the extent of tumor and brain distribution of two anticancer drugs, doxorubicin and paclitaxel, used in the treatment of metastatic breast cancer.

Methods: Brain-seeking MDA-MB-231BR human breast carcinoma cells transfected with eGFP were injected into the left ventricle of immune-compromised NuNu mice. Tumors were allowed to seed and grow within the central nervous system for 4–6 weeks. Mice were anesthetized and administered radiolabeled paclitaxel or doxorubicin. At 30 minutes after injection, the brain was removed from the cranium, frozen in isopentane, and cut into tissue sections. Sections were mounted on glass slides and processed for fluorescent analysis, autoradiography, and/or tissue staining.

Results: Drug uptake differed among tumors with elevated uptake in some but not all large tumors (>1–2 mm diameter), whereas distribution in smaller micrometastases (<500 µm) was typically comparable to normal brain. When elevated tumor drug distribution was observed, it generally correlated with increased blood-tumor barrier permeability to passive markers. The magnitude of increase in drug distribution varied from 3–10-fold. Distribution of drugs within leaky tumors was heterogeneous with the tumor core showing greater distribution than brain adjacent to tumor. Pharmacokinetic modeling was performed to assess the impact of a 3–10-fold increase in blood-tumor barrier permeability of tumor drug exposure.

Conclusion: The results demonstrate variable paclitaxel and doxorubicin distribution in this brain metastatic model of breast cancer where tumor cells were delivered hematogenously to the central nervous system. No alteration was observed in drug exposure to micrometastases, whereas some larger tumors showed increased exposure that differed within the site of the tumor.

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P39-20: PRECLINICAL STUDIES IN SUPPORT OF THE USE OF VORINOSTAT (SAHA) FOR THE TREATMENT OF BRAIN METASTASES OF BREAST CANCER

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The incidence of brain metastases in breast cancer patients is increasing in part because chemo- and molecular therapies improve systemic survival. Few therapeutic strategies exist for the treatment of metastatic tumor cells in the brain where the blood-brain barrier can severely limit drug access. We have established a xenograft mouse model of breast cancer brain metastasis for preclinical testing of potential therapeutics. The model uses an EGFP-labeled brain metastatic subline (231-BR) of the MDA-MB-231 breast cancer cell line. This model system represents the triple negative (ER-, PR-, Her-2 unamplified) or basal breast cancer phenotype. Up to 30% of triple negative metastatic breast cancer patients develop brain metastasis. Herein, we used this model to test the efficacy of histone deacetylase (HDAC) inhibitors for the prevention and treatment of breast cancer brain metastasis. In vitro, the HDAC inhibitors TSA, valproic acid, depsipeptide, and Vorinostat (SAHA) all inhibited the growth of 231-BR cells with a concomitant increase in histone H3/H4 acetylation. At concentrations of less than the IC₅₀, HDAC inhibition drastically reduced basal and chemoattractant-stimulated motility of 231-BR cells in a Boyden chamber assay. In addition, Vorinostat induced apoptosis in 231-BR cells in a dose- and time-dependent manner. Next, Vorinostat was also tested in vivo in prevention and treatment models of breast cancer brain metastasis. In the prevention model, treatment was initiated 3 days after intracardiac injection of 1.0 x 10⁵ 231-BR cells. Compared with the untreated control group, Vorinostat at 150 mg/kg qd inhibited the development of large (>50 µm²) brain metastases by approximately 50% (p=0.0002). In the treatment model, Vorinostat (SAHA) dosing was initiated 3, 7, and 14 days, respectively, after intracardiac injection of 1.75x10⁵ 231-BR cells. Compared with the untreated control group, there was a 30% reduction in micrometastases (p=0.017) and a greater than 60% reduction in the number large metastases (p<0.0001) when treatment was initiated on day 3, confirming and extending the results of the prevention model. There was also a significant reduction in large metastases (p=0.008) when treatment was initiated on day 7. However, there was no effect of Vorinostat (SAHA) when treatment was initiated 14 days after injection of 231-BR cells. These data suggest a new drug for the prevention of brain metastases of breast cancer and indicate a therapeutic window for treatment. Analysis to determine the molecular mechanism of action of Vorinostat (SAHA) in this model is ongoing.

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P39-21: LAPATINIB PREVENTS METASTATIC COLONIZATION OF EGFR+ AND HER-2+ BREAST CANCER CELLS

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Central nervous system or brain metastases occur in 10%–20% of metastatic breast cancer patients but in upwards of 35% of patients whose tumors overexpress Her-2. No effective treatment exists, and the progress to cure brain metastasis has been limited by the lack of suitable cell and animal models. Herein, we determined the efficacy of the dual EGFR/Her-2 tyrosine kinase inhibitor lapatinib on the brain metastatic colonization of a human breast carcinoma cell line. An EGFP-labeled, brain-seeking derivative of the human MDA-MB-231 cell line (231-BR) endogenously overexpressed EGFR and was transfected with a control vector or Her-2 cDNA to express one or both of lapatinib's targets.

In vitro, lapatinib inhibited phosphorylation of EGFR and Her-2, leading to disruption of the MAPK, AKT, and PLCγ1 pathways. Lapatinib inhibited the proliferation of 231-BR-Her-2 cells and vector-transfected controls cultured for 96 hr with IC₅₀s of 7.5 and 8.5 mM, respectively. Motility of the Her-2 cell line was statistically significantly inhibited at 1 µM and 3 µM treatment (p=0.030 and p=0.009, respectively). However, vector control cells showed significant inhibition of motility only at 3 µM lapatinib treatment (p=0.013). Using siRNA to EGFR, our data indicate that the antiproliferative activity of lapatinib toward EGFR and Her-2 in the 231-BR cells is comparable, and expression of two targets may produce increased sensitivity to lapatinib treatment.

In vivo, 231-BR control cells showed a mean 3.36 (95% CI 2.73 to 3.98) clinical metastases per brain section in the vehicle treated mice. The mean was unaffected by 30 mg/kg lapatinib but declined 54% to 1.56 (0.94 to 2.17) with 100 mg/kg lapatinib (p=0.0001). The Her-2-overexpressing cells produced 6.83 (5.86 to 7.79) clinical metastases per section. Treatment with 30 mg/kg lapatinib resulted in a 53% decline to 3.21 (2.31 to 4.11) clinical metastases (p<0.0001) while treatment with 100 mg/kg resulted in a 50% decline to 3.44 (2.55 to 4.32) metastases (P<0.0001). Immunohistochemical analysis of residual brain metastases showed significant reduced pHer-2 expression (P< 0.0001 at high dose [100 mg/kg]) in the Her-2 cell line. However, no significant effect of lapatinib on low (0-1+) pEGFR staining was observed in any treatment group for either the Her-2 or vector control cell lines. Immunohistochemical studies are ongoing for other molecules of interest in the signaling pathway. The pharmacodynamic effects of lapatinib on EGFR activation were distinct in vitro and in vivo. This inability to shut down EGFR phosphorylation may contribute to drug resistance. These data suggest that a brain-permeable EGFR inhibitor may constitute a future rational combination for augmentation of lapatinib's preventative activity.

Lapatinib represents the first Her-2-directed drug to be validated in a preclinical model for activity against brain metastases, suggesting clinical activity in the prevention setting.

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P39-22: BRAINMETSBC.ORG: EVALUATION OF A NEW RESOURCE FOR BREAST CANCER PATIENTS WITH BRAIN METASTASES

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Introduction: As improved treatments control extracranial disease and extend survival, more women with metastatic breast cancer face a diagnosis of CNS metastases. An estimated 10% to 16% of all breast cancer patients whose disease has spread develop brain metastases; for those whose cancer is HER2-positive, a brain metastasis incidence of 25% to 48% has been reported. To address this growing crisis, a 5-year Center of Excellence (COE) comprised of scientists, translational researchers, and advocates was funded by the Department of Defense Breast Cancer Research Program.

Studies have shown that women with metastatic breast cancer need dedicated resources. In December 2007, patient advocates working with the COE launched a new consumer website, BRAINMETSBC.ORG, to offer information and support about brain and other CNS metastases to patients and caregivers. The present survey assesses how well BRAINMETSBC.ORG meets this need.

Methods: Visitors to BRAINMETSBC.ORG were invited to fill out an 18-question anonymous survey examining the helpfulness, clarity, and design of this website. Responses were elicited from patients and caregivers dealing with brain metastases, metastatic patients not yet facing this diagnosis, patient advocates, and health care providers.

Results: There were 84 survey responses within the first 2 weeks of website launch. Nearly half (47%) were patients dealing with brain metastases or their caregivers. Respondents were young with 52% aged 50–59, 19% aged 40–49, and 24% aged 30–39. Nearly half (46%) had been living with metastatic breast cancer for 3 to 5 years while an equal proportion (43%) had been dealing with brain metastases for less than 1 year.

Most patients and caregivers (89%) said they would have found the website helpful when first diagnosed with brain metastases; 86% found it helpful at the present time. Disease and treatment information was rated as most helpful, followed by patient experiences, ongoing research efforts, researcher interviews, and clinical trials listing. Almost all (90%) said they knew more about CNS metastases after visiting the website. Most patients and caregivers (64%) said they found it reassuring to know that some women can live with brain mets. Many felt less worried (52%) and more in control (41%) after reading the website, by contrast with 9% who felt this kind of information was better on a “need to know” basis.

The patient advocates and health care providers who rated the website said it would be very (78%) or somewhat (22%) helpful to them in working with patients and families. Almost all survey respondents found the medical information clearly presented (93%) and said the website was well organized (89%) and easy to navigate (91). The most common expressed need (43%) was for more treatment information.

Conclusions: Most patients and caregivers, advocates, and health care providers highly rated BRAINMETSBC.ORG. Those already diagnosed wished this resource had been there for them while those worried about getting brain metastases felt less fearful, armed with medically reviewed information, and stories from women who have been there. The in-depth information provided and the reassurances from patients living with the disease were cited as valuable as was learning that ongoing research is being actively pursued. Treatment updates and refinements will be crucial to the ongoing success of this website.

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P39-23: TUMOR CELL:MICROENVIRONMENTAL INTERACTION IN A XENOGRAFT MODEL OF BREAST CANCER BRAIN METASTASIS AND PIGMENT EPITHELIUM-DERIVED FACTOR AS A SUPPRESSOR OF BRAIN METASTASIS

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Brain metastases occur in 10%–20% of metastatic breast cancer patients. The 1-year survival rate is approximately 20%. We have developed a model system for the study of breast cancer brain metastasis using an EGFP-labeled “brain-seeking” derivative of the human MDA-MB-231 breast carcinoma cell line (231BR) in the mouse. One month after intracardiac injection of 231BR cells, metastases are observed in the brain of nude mice. An analysis of the pathology of our xenograft model, reported here, improves our understanding of metastatic progression in the brain. Tumors form in both the meninges and brain parenchyma. Metastases in all locations show high levels of proliferation (~53% by Ki67 staining); however, most parenchymal tumor growth manifests as clusters of neighboring “micrometastases.” A clonal analysis was performed by co-injecting a mixed population of tumor cells expressing one of several fluorescent proteins. The results proved that single tumor cells give rise to all the “micrometastases” in a given cluster. This results from preferential growth and migration along neighboring branches of the brain vasculature. Further analysis of the brain tissue surrounding metastases shows a massive inflammatory response with extensive reactive gliosis around metastases. Glial cells promote anchorage-independent growth of 231BR cells in vitro, suggesting that they secrete factors conducive to metastatic growth. We have chosen to pursue pigment epithelium-derived factor (PEDF, also known as SerpinF1) as a potential suppressor of brain metastasis. PEDF was downregulated in human brain metastases relative to the primary tumors (~2-fold), suggesting that it may have a negative impact on brain metastatic progression. PEDF, a secreted factor, has been shown to act as a tumor suppressor, has strong antiangiogenic activity, and affects the proliferation/viability/differentiation of neural tissue. Since metastases in our model system grow in close association with both the brain vasculature and with surrounding glial cells, we hypothesized that the multiple roles of PEDF might make it a potent agent that targets not only tumor cells but also the brain microenvironment. To investigate its effect on metastatic progression, PEDF was overexpressed (2- to 5-fold) in our 231-BR cells. In vitro, PEDF expression strongly inhibited (greater than 50-fold) anchorage-independent growth of 231-BR cells in soft agar. The in vivo effects of PEDF on angiogenesis and metastatic outgrowth of 231BR cells in the brain are currently under investigation.

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P39-24: ABSTRACT WITHDRAWN

P39-25: HEXOKINASE-2 IS A KEY THERAPEUTIC TARGET IN BRAIN METASTASIS DERIVED FROM BREAST CANCER

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The prevalence of brain metastasis from breast cancer is increasing. Current treatment options are limited and have no impact on survival. We are evaluating several potential therapeutic targets identified from cDNA microarray analysis of surgically resected human brain metastases. One of these, hexokinase II (HK-2), was validated using quantitative RT-PCR and showed an approximately 2-fold upregulation ($p=0.01$) in brain metastases ($n=8$) compared to unmatched primary invasive ductal breast carcinomas ($n=8$). Since HK-2 is not normally found in brain, this suggests that this isoenzyme may be a selective target for metastatic disease in this organ. HK-2 is upregulated in many tumors and belongs to a family of four hexokinases that play a vital role in the generation of ATP as they catalyze the first step of the glycolytic pathway converting glucose to glucose-6-phosphate. Production of this substrate also drives the pentose phosphate pathway, providing many components required for cell growth. In addition, through mitochondrial binding, HK-2 inhibits apoptosis. We therefore hypothesized that HK-2 would play a key role in cell growth and survival in brain metastasis derived

from breast cancer and that its level of expression would affect overall survival. To test this hypothesis, we examined survival curves in patients that were divided into one of two groups based on the degree of HK-2 expression in brain metastases as determined by immunohistochemistry. Median survival for patients ($n=94$) with a high level of HK-2 was 9.6 months post-surgery as compared to a median survival of 17.5 months in patients ($n=19$) with a low level of HK-2 ($p=0.0275$ by two-tailed log rank test). To further validate HK-2 as a therapeutic target and evaluate the functional role of HK-2 in brain metastases, we are using a human breast cancer cell subline that is selectively metastatic to the brain (MDA-MB-231BR). These cells were transduced using VSV-g pseudotyped lentiviral particles carrying a shRNA targeting either HK-2 or a nontarget control (Sigma-Aldrich). Knockdown of HK-2 was verified by western blotting and QT-PCR. Cell growth is measured using 5-bromo-2'-deoxy-uridine labeling. Knockdown of HK-2 expression by 95% inhibited tumor cell proliferation by 40% ($p=0.0012$ by two-tailed t test) under normal growth conditions at 5 days. These data suggest that overexpression of HK-2 is associated with poorer overall patient survival because it plays a key role in the proliferation and long-term survival of metastatic cells in brain. Thus, HK-2 is likely an important therapeutic target in brain metastasis derived from breast cancer.

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P39-26: INHIBITION OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 ACTIVATION SUPPRESSES THE BRAIN METASTASES OF MDA-MB-231-BR CELLS IN NUDE MICE

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Brain metastasis is a major cause of morbidity and mortality in patients with breast cancer. The molecular changes that lead to brain metastasis remain poorly understood. In the present study, we found that the level of signal transducer and activator of transcription 3 (Stat3) activation was increased in human brain-metastatic breast cancer cells when compared with that in primary breast cancer cells. To investigate the role of Stat3 activation in brain metastases of breast cancer, we used SOCS-1 (suppressor of cytokine signaling-1) protein to inhibit Stat3 activation in MDA-MB-231BR brain metastatic cells. MDA-MB-231BR cells were transfected with low (2-fold) or high (6-fold) levels of SOCS-1. SOCS-1-overexpressing 231-BR cell lines have decreased levels of phospho-Stat3 but not total Stat3. In vitro, SOCS-1-overexpressing 231-BR cell lines have similar anchorage-dependent growth rates as control transfectants, but anchorage-independent growth of the cells was significantly decreased compared to control transfectants. In vivo, high SOCS-1-overexpressing 231-BR cell lines did not form subcutaneous tumors in nude mice whereas control transfectants formed aggressively growing subcutaneous tumors. Moreover, in a model of brain metastasis, high SOCS-1-overexpressing 231-BR cell lines failed to produce brain metastasis in nude mice, but control transfectants produced brain metastases in all of the mice injected. Mechanistically, overexpression of SOCS-1 significantly inhibited the expression of Stat3 target genes cyclin D1, survivin, and vascular endothelial growth factor genes. These data suggest that inhibition of Stat3 activation by SOCS-1 suppressed brain metastasis of breast cancer. Therefore, Stat3 activation might be a new potential target for therapy of human breast cancer brain metastases.

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P39-27: EX VIVO SINGLE CELL MRI WITH FIESTA: QUANTITATIVE BENEFITS OF 3T OVER 1.5T

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Metastatic brain tumors arise in 15% of metastatic breast cancer patients and lead to 1-year survival rates of only ~20%. This is attributed, in part, to the fact that surgery and radiation can only eradicate ~90% of a metastatic brain tumor with 10% remaining in the form of micro-metastases or single dormant cells causing patient relapse. Recently, we showed for the first time that individual iron-labeled cells can be detected as signal voids in vivo in mouse brain using a 1.5 Tesla (T) magnetic resonance imaging (MRI) scanner equipped with a custom-built gradient coil insert and a 3DFIESTA pulse sequence (three-dimensional fast imaging steady-state acquisition). In this study we investigate the feasibility of using 3DFIESTA for single cell detection at the higher magnetic field strength of 3T.

Human breast carcinoma cells, MDA-MB-231BR, were labeled with iron-oxide particles and 30,000 labeled cells were injected into the left ventricle for systemic distribution of cells. The mouse heads were scanned ex vivo 3 days post-injection using identical 1.5 and 3T MR systems. First, two methods for reducing banding artifacts, the main

obstacle faced with FIESTA imaging at higher field strengths, were tested at 3T: repetition time (TR) minimization and phase cycling. Then, the effect of field strength on signal void detection was investigated by comparing MR images acquired with identical parameters at 1.5 and 3T. Two comparisons were then conducted in order to optimize signal void detection at 3T: the first tested 4 different bandwidths (BW: ± 23 , 13, 8, 5 kHz; corresponding to TR: 7, 10, 15, and 22 ms) while holding resolution and scan time constant. The second compared our standard resolution of $100 \times 100 \times 200 \mu\text{m}^3$ with a higher one of $100 \times 100 \times 100 \mu\text{m}^3$ while matching signal-to-noise (SNR) by increasing scan time by fourfold. For each image data set, the number of signal voids was counted in 30 image slices, and the signal void contrast was measured by calculating the fractional signal loss (DS/S).

Minimizing TR, accomplished by increasing BW, was successful at reducing banding artifacts at 3T; one drawback was the decreased SNR associated with large BWs. Phase cycling was found to effectively reduce banding artifact without any adverse effects on SNR; for this reason, it was implemented in all further imaging. In the field strength comparison, double the number of signal voids was counted at 3T compared to 1.5T. In the bandwidth comparison, reducing BW was found to increase signal voids' DS/S, thus improving signal void detection. The number of signal voids detected, however, increased with decreased BW until it reached a plateau at $\pm 8\text{kHz}$; decreasing BW further increased the amount of motion artifact. In the resolution comparison, DS/S was higher at the higher resolution; however, the number of signal voids was not different between the two resolutions.

This study has demonstrated the feasibility of 3DFIESTA imaging for cell detection on a 3T clinical scanner. The optimal 3T 3DFIESTA scanning protocol for detecting individual signal voids produced by iron-labeled cells in ex vivo mouse brain images is: BW of $\pm 8\text{kHz}$, SNR of ~ 100 , and resolution of $100 \times 100 \times 200 \mu\text{m}^3$. This study revealed that $\sim 60\%$ of the signal voids were not detectable with the 1.5T protocol compared to the optimized 3T protocol. Future experiments will utilize the optimized 3T FIESTA imaging protocol to quantify in vivo cell detection.

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P39-28: MONITORING THE FATE OF SOLITARY METASTATIC CELLS IN MOUSE BRAIN USING MAGNETIC RESONANCE IMAGING

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Metastatic brain tumors arise in 15% of metastatic breast cancer patients, leading to a 1-year survival rate of only 20%. This is attributed to failure of treatment to eradicate all metastatic tumor cells. Recently we showed for the first time that individual iron-labeled cells can be detected as signal voids in vivo in mouse brain using a micro-imaging system on a magnetic resonance imaging (MRI) scanner. However, the effect of proliferation, dormancy, and apoptosis on MR signal voids in vivo is not fully understood. This study was thus undertaken to model the effects of dormancy and apoptosis on MR signal voids.

MDA-MB-231BR brain metastasizing cells were labeled with micron-sized iron-oxide particles (MPIO) and exposed in vitro to regular growth medium ("proliferating" cells), formalin ("non-proliferating" cells), or doxorubicin ("apoptotic" cells). 10^5 labeled cells from each group were injected into the left ventricle of the heart using three separate experimental groups of mice. In vivo images of the brain were acquired on days 1, 7, 14, 21, and 28 post injection using a 3T clinical MRI scanner equipped with a customized gradient insert, 3DFIESTA (three-dimensional fast imaging steady-state acquisition) pulse sequence, and $100 \times 100 \times 200 \text{mm}^3$ spatial resolution in 24 minutes. For each image data set, the number of signal voids, corresponding to iron-labeled cells, was counted in 30 image slices.

Regions of signal void were detected in the brains of all mice. In mice injected with "proliferating" cells, the number of signal voids initially declined quickly (38% remaining at day 3), followed by a period of slow signal void loss (14% remaining at day 28); micrometastases were detected by day 21. This is consistent with the metastatic growth model in which subpopulations of dormant, proliferating, and apoptotic cells co-exist. Persistent signal voids would thus be due to dormant cells while signal void loss is due to apoptosis or proliferation of cells, resulting in loss or dilution of iron label. In mice injected with "apoptotic" cells, the number of voids declined rapidly with only 9% remaining by day 7, indicating apoptosis leads to a quick loss of signal voids. In mice injected with "non-proliferating" (fixed) cells, the number of signal voids declined slowly with $>50\%$ of voids remaining at 14 days, consistent with a homogenous population of cells/voids unable to proliferate or undergo apoptosis. No tumors developed in mice injected with fixed or doxorubicin-treated cells.

Here we were able to observe differences between live proliferating, non-proliferating (fixed), and apoptotic cancer cells in vivo over time using cellular MRI techniques. We found that signal voids from apoptotic iron-labeled cells were rapidly lost with no sub-

sequent tumors developing. This contrasted with non-proliferating cells in which a slow linear decrease in signal voids was observed. In the heterogeneous "proliferating" cell population, characteristics of dormant (persistent voids), apoptotic (signal void loss), and proliferating cells (void loss followed by appearance of micrometastases) were observed. These results clarify the effect of proliferation, dormancy, and apoptosis of cancer cells on MR signal voids and will be useful in more detailed studies of the effect of treatment on solitary metastatic breast cancer cells in the brain.

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P39-29: AN IN VITRO MODEL OF METASTATIC BREAST CANCER CELL EXTRAVASATION THROUGH ENDOTHELIAL CELL MONOLAYERS

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Metastasis is the principal cause of death from breast cancer. The extravasation of cancer cells from the circulation is thought to be a critical step in metastasis and is difficult to model in vitro. We are using electric cell substrate impedance sensing (ECIS) as a model of cancer cell extravasation through monolayers of endothelial cells. This assay is based on the measurement of changes in resistance across a $250 \mu\text{m}$ electrode to the flow of small AC currents. When cells are plated onto electrodes in modified culture slides, current flow is impeded proportional to the adhesion and attachment of the cells. Extravasation of cells through an endothelial monolayer plated onto the electrodes is detected by a drop in resistance values, corresponding to the increased flow of current as the endothelial cells retract. The assay was used to compare the extravasation abilities of the human breast cancer cell line MDA-MB-231 and isogenic variants established from metastases in the brain and lungs of nude mice. Compared with the original cell line and the lung metastasis-derived variant, the brain metastasis-derived 231-BR3 variant had greater ability to form experimental brain metastases. The 231-BR3 cells also had greater potential to extravasate through a monolayer of rat brain endothelial cells, indicated by a sharp decline in ECIS resistance values. The 231-BR3 cells release more vascular endothelial growth factor (VEGF) than MDA-MB-231 cells. VEGF promotes vascular permeability, disrupts endothelial barrier function, and thus may facilitate the extravasation of metastatic cells. Expression of VEGF in 231-BR3 cells was significantly suppressed using siRNA (control = $1,350 \text{ pg/mL}$ versus siRNA treated = 25 pg/mL , measured by ELISA). The cells with silenced VEGF showed reduced extravasation in the ECIS assay. Pre-treatment of the endothelial cell layer for 1 hr with an inhibitor of VEGF-receptor tyrosine kinases (PTK787) also attenuated the extravasation of 231-BR3 cells. The results suggest that high VEGF expression contributed to the enhanced extravasation ability of 231-BR3 cells. Additional genes that are differentially expressed by the 231-BR3 cells compared with the nonselected cell line have been identified, including MMP-1, IL-8, and oncostatin M/IL-6 signal transducer. Ongoing experiments are investigating the contribution of these genes to the extravasation ability of the brain metastasis-selected cells.

Conclusions: The ECIS assay can provide novel information about interactions between metastatic cells and endothelial cells and the molecules involved in the process of extravasation. The assay can be used as a tool to identify molecular regulators of breast cancer cell extravasation.

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P39-30: EFFICACY OF ENHANCED DELIVERY OF TRASTUZUMAB-AURISTATIN IMMUNOCONJUGATES IN A RAT MODEL OF BREAST CANCER BRAIN METASTASIS

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Introduction: Brain metastasis develops in 20%–30% of patients with breast cancer. Metastases in the central nervous system are difficult to treat at least in part because the blood-brain barrier (BBB) limits delivery of therapeutic agents to the brain, particularly high molecular weight agents such as monoclonal antibodies (mAb). Trastuzumab (HerceptinTM) anti-HER2 mAb shows clinical efficacy in subjects with breast cancer but has not been evaluated in brain metastases. We tested whether immunoconjugates of trastuzumab with highly toxic auristatin chemotherapy were effective against a nude rat model of breast cancer brain metastases when delivery to the brain was enhanced with osmotic BBB disruption (BBBD).

Methods: A human MDA-MB-231BR breast carcinoma cell line expressing high levels of HER2 was implanted intracerebrally in female nude rats. Trastuzumab and three trastuzumab-auristatin immunoconjugates (obtained from Genentech) were administered intra-arterially (IA) immediately after BBBD on day 8 after tumor implantation. Magnetic resonance imaging (MRI) was performed to evaluate tumor response, and brains were harvested on day 17 for volumetric analysis ($n = 4$ per group). Subsequently, lower doses of the most effective immunoconjugate (Herceptin-

MC-vc-PAB-MMAE [HC-AE]) were tested in the same experimental paradigm. An MRI and survival study to evaluate the efficacy of HC-AE in breast cancer brain metastases expressing high or low levels of HER2 is ongoing.

Results: In untreated animals, brain tumor volume was 9.1 ± 3.0 mm³ (mean \pm SEM) at the beginning of study (day 8), and 86.4 ± 23.6 mm³ at the end of study (day 17). Trastuzumab (10 mg/kg IA/BBBD) was ineffective in this model with a final tumor volume of 78.8 ± 17.5 mm³. All three trastuzumab-aurotin immunconjugates were toxic to HER2-expressing MDA-MB-231BR cells in vitro and in vivo. The HC-AE conjugate provided the best efficacy in vivo with a tumor volume of 25.8 ± 5.2 mm³ ($p < 0.05$ compared to untreated control). In the second study with lower doses, 3 mg/kg of HC-AE given IA/BBBD significantly reduced tumor growth, but 1 mg/kg immunconjugate did not.

Conclusion: Enhanced delivery of trastuzumab alone was ineffective in this brain tumor model. Delivery of trastuzumab-aurotin immunconjugate across the BBB was effective against breast cancer brain metastases.

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P39-31: ErbB2 OVEREXPRESSION AND PTEN-LOSS COOPERATE TO FURTHER ENHANCE BREAST CANCER BRAIN METASTASIS

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Higher ErbB2 gene amplification was detected in brain metastases compared to primary breast tumors (37% versus 8%). Recently, ErbB2/HER-2 overexpression has been shown to increase the metastatic outgrowth of breast cancer cells in the brain. Interestingly, a higher rate of PTEN loss of heterozygosity (LOH) in breast cancer-derived brain metastases was detected compared to primary breast tumors. We have performed immunohistochemistry (IHC) analysis of PTEN protein expression in 146 primary breast tumors and nonmatched 131 breast cancer brain metastases. We found a significant higher rate of PTEN-loss in brain metastases (71%) than primary tumors (38%). This correlation between PTEN deficiency and brain metastases indicated that PTEN-loss may favor breast cancer metastasis to the brain. Additionally, we have demonstrated that PTEN activation contributes to tumor inhibition by trastuzumab (Herceptin®), and loss of PTEN predicts trastuzumab resistance in patients. Our data also indicate a functional link between PTEN and ErbB2 in breast cancer progression. We hypothesized that PTEN-loss cooperates with ErbB2 to further enhance ErbB2-overexpressing breast cancer metastasis to the brain. To test our hypothesis, we used an MDA-MB-231 brain-seeking subline 231-BR.HER2 that overexpresses ErbB2/HER2 as our study model. We knocked down PTEN expression in 231-BR.HER2 cells by stable transfection of PTEN shRNA as well as scrambled control vector. Cell proliferation and metastatic properties of PTEN-deficient and HER2-overexpressing cells were analyzed in vitro. Brain metastatic activities of 231-BR.HER2.PTENshRNA cells were also investigated in an in vivo brain metastasis animal model with carotid artery injection of cancer cells. In in vitro assays, PTEN-deficient and HER2-overexpressing cells had more aggressive metastatic properties than PTEN-normal and HER2-overexpressing cells as demonstrated by increased cell migration toward brain endothelial cells and higher trans-endothelial cell invasion activities. In addition, PTEN-deficient and HER2-overexpressing cells also exhibited a survival advantage over the PTEN-normal and HER2-overexpressing cells in vitro. In the experimentally induced brain metastasis mouse model, mice injected with PTEN-deficient and HER2-overexpressing cells exhibited a slightly reduced overall survival compared to PTEN-normal and HER2-overexpressing group. More significantly, PTEN-deficient and HER2-overexpressing cells yielded more micro- (<50 μ m) and macro- (>50 μ m) metastases over the PTEN-normal and HER2-overexpressing group in the brain metastasis animal model. Collectively, our data indicated that ErbB2 overexpression and PTEN-loss can cooperate to further enhance breast cancer brain metastasis. The findings have a therapeutic implication for breast cancer brain metastasis.

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P39-32: METASTASIZING BREAST CARCINOMA TO THE BRAIN: A CLINICOPATHOLOGIC AND IMMUNOHISTOCHEMICAL STUDY OF 14 MATCHED PAIRS

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Brain metastasizing breast cancer is the second most common cause of brain metastases. The frequency of brain metastasis ranges between 10%–40% of breast cancers. Hormonal status of the primary malignancy is thought to play a role in determining which tumors eventually will show brain metastasis. In this study, we examined

14 pairs of matched primary breast carcinomas and their respective brain metastases for various clinicopathologic parameters and performed immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PR), Her-2/neu, and epidermal growth factor receptor (EGFR). The mean age at diagnosis of breast primary and the subsequent brain metastases was 54.8 years (36–74) and 57.7 years (39–75), respectively. The mean interval between the diagnosis of breast carcinoma and the brain metastasis was 32 months (4 months–8 years). All 14 breast primary cancers were of the invasive ductal type and over half (8/14) were high grade (SBR III/III). Nine (64%) of the matched pairs were ER negative and 10 (71%) were PR negative, but only 7 (50%) were both ER/PR negative. Three (21%) pairs were found to be Her2/neu positive by immunohistochemistry. Overall, 5 (36%) matched pairs were “triple negative.” Diffuse and strong EGFR immunoreactivity was observed in 4 primary breast cancers and only 2 brain metastases. Both EGFR-positive brain metastases had EGFR-positive primaries. EGFR-positive pairs were more frequently “triple negative” or in one case, ER/PR negative and Her2/neu positive. Her2/neu analysis by FISH is under way.

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P39-33: BLOOD-TUMOR BARRIER PERMEABILITY AND VASCULARITY IN A MODEL OF BRAIN METASTASES OF BREAST CANCER

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Introduction: Drug treatment of brain metastases presents a special problem because of the limiting properties of the blood-brain barrier. Tumor vascularity and permeability have been implicated as important factors in tumor development and susceptibility to chemotherapeutics. Most animal models of brain metastatic cancer rely upon direct intracranial implantation of tumor cells via injection. This process circumvents several steps in tumor implantation and development in brain. To overcome these limitations, we studied vascular permeability and density in a novel model where tumor cells were delivered via the circulation.

Methods: Brain-seeking MDA-MB-231BR human breast carcinoma cells transfected with eGFP were injected into the left ventricle of immune-compromised NuNu mice. Tumors were allowed to seed and grow within the central nervous system for 4–6 weeks. Mice were anesthetized and administered small (<5kD) and large (>60kD) molecular weight vascular markers to measure blood-tumor barrier permeability and vascular density. At predetermined times, the brain was removed from the cranium, frozen in isopentane, and cut into tissue sections. Sections were mounted on glass slides and processed for fluorescent analysis, autoradiography, and/or tissue staining.

Results: Clusters of micrometastases of different sizes (50 μ m–2.5 mm diameter) were observed within brain at 4–6 weeks using eGFP and co-staining with antihuman cytokeratin antibody as identification markers. In most metastases, vascular density was reduced but vascular volume increased. Vascular barrier permeability differed among tumors and depended upon marker molecular weight and tumor size. In most small micrometastases (diameter <500 μ m), vascular permeability was low and comparable to adjacent normal brain. In larger tumors, significant heterogeneity was found with tumor permeability rising 5–10-fold in some tumors >1 mm diameter and others remaining unchanged. Distribution of tracers within leaky tumors was heterogeneous with the tumor core generally showing greater distribution than the tumor periphery.

Conclusion: The results demonstrate variable integrity of the blood-tumor barrier in this brain metastatic model of breast cancer where tumor cells were delivered hematogenously to the central nervous system. In particular, the results highlight the integrity of the blood-brain barrier to drug delivery in micrometastases and smaller tumors.

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P39-34: ROLE OF YKL-40 IN MESENCHYMAL TRANSITION AND BREAST TO BRAIN METASTASIS

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YKL-40 was identified by our group to be highly expressed in a subset of glioblastoma patients that exhibit poor overall survival and resistance to radiation treatment. The expression of YKL-40 was also found to be associated with tumors that express a mesenchymal group gene signature. Since epithelial to mesenchymal transition is a well-defined phenomenon in breast cancer that correlates with a metastatic phenotype, we sought to investigate the role of YKL-40 in these tumors. MCF-7 cells overexpressing YKL-40 exhibited decreased cell-cell contacts and decreased membranous E-cadherin and beta (β)-catenin expression. As a consequence, β -catenin was increased in the

cytoplasm and nucleus, and c-myc a known target of β -catenin signaling was increased with YKL-40 expression. The mesenchymal transition however did not involve induction of markers such as vimentin and fibronectin. MCF-7 clones expressing YKL-40 also exhibited increased migration and invasion in transwell assays. We screened for YKL-40 expression in four mouse mammary tumor cell lines, 67NR, 168FARN, 4TO7, and 4T1, with increasing metastatic ability and found highest levels of YKL-40 in 4T1 cells that are capable of forming visible nodular lung metastasis but very low expression in 67NR cells that form only primary tumors without any detectable metastasis. We next analyzed tissue samples from 123 women who underwent surgical resection of metastasis to the brain parenchyma (BM) with histologically proven breast carcinoma at MDACC between 1984–2004 and were examined for estrogen receptor- α (ER) by IHC and HER2 by FISH or IHC, as well as YKL-40 (IHC). The ER-/Her2- profile was seen in 43% of tumors. The median post-neurosurgical survival (PNS) was 11.2 months for the 112 patients with no pre-craniotomy brain radiation. YKL-40 positivity was highly associated with the ER-/Her2- profile, as the majority of YKL-40-positive cases were negative for both markers. YKL-40 positivity was associated with both a shorter interval between breast primary diagnosis and neurosurgery, as well as shorter post-neurosurgical survival. In summary, YKL-40 is expressed in a minority of brain-metastatic breast cancers and is associated with the ER-/Her2-profile, and poorer patient outcome. We hypothesize that YKL-40 mediates these effects, at least in part, by inducing β -catenin signaling in breast cancer.

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P39-35: PHYSICAL AND MENTAL HEALTH STATUS OF YOUNG BREAST CANCER PATIENTS DURING THE FIRST 3 YEARS FOLLOWING DIAGNOSIS

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Background and Purpose: About 15% of the incident cases of breast cancer occur in women of childbearing age and the majority will be long-term survivors. Most young women with invasive breast cancer will undergo adjuvant chemotherapy and half or greater will undergo premature menopause. The health-related quality of life (HRQL) of these young patients may be compromised by the treatments received and/or premature menopause, with symptoms such as hot flashes and night sweats. Very little is known about the HRQL of young cancer patients by menopausal status following breast cancer therapy. The purpose of this study was to examine the impact of demographic and treatment-related factors on young patients' physical and mental health status during the first three years following diagnosis.

Sample and Method: Participants were 627 patients recruited to the Menstrual Cycle Maintenance and Quality of Life Study between 1998 and 2002. Patients were 18–45 years old with regular menstrual cycles at the time of diagnosis with a stage 1–3 breast cancer. Demographic (age, race, education, marital status) and treatment-related variables (stage at diagnosis, type of surgery, treatments received, menstrual bleeding status during follow-up, hot flashes, night sweats, body mass index) were used in linear mixed models to predict participants' physical and mental health status during the first three years following diagnosis. The dependent variables were the PSC-12 and the MSC-12 summary scores of the SF-12.

Results: Variables significantly predictive of worse physical functioning during the first 3 years following diagnosis were being Asian as compared to a non-Hispanic White ($p=0.0095$), a body mass index (BMI) in the obese range (> 30) as compared to the normal (< 25) or overweight ranges ($25-29$) ($p<0.0001$), and taking tamoxifen ($p=0.038$). Having moderate-to-severe hot flashes compared to no hot flashes was borderline significant for reduced physical functioning ($p=.0519$). There was also a significant time by chemotherapy interaction in that effects of chemotherapy on physical functioning lessened as the time from chemotherapy increased ($p<.0001$). Variables predictive of better physical functioning were having menstrual bleeding in the preceding 3 month period ($p=0.04$), a higher number of positive nodes at diagnosis ($p<.0001$), and a greater number of months since diagnosis ($p=0.0005$). Regarding the participants' mental health status, having greater than a high school education ($p=0.0275$) and being divorced or widowed as compared to married ($p=0.039$) were significantly related to worse mental functioning. Greater time since diagnosis, however, was significantly related to better mental health functioning ($p=0.018$). No other demographic or treatment-related variables were significantly related to mental functioning.

Conclusions: Both the physical and mental health statuses of these young patients improved with time during the first three years following diagnosis. The participants' physical health was more impacted by treatment-related variables than the participants' mental health status. Survivors who received adjuvant chemotherapy and/or hormonal therapy may need continuing interventions to assist them in managing their symptoms and improving their HRQL.

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P39-36: EXPLAINING AGE ASSOCIATED DIFFERENCES IN PSYCHOLOGICAL MORBIDITY FOLLOWING A BREAST CANCER DIAGNOSIS

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Objective: Research consistently finds that women diagnosed with breast cancer at a younger age have significantly greater psychological morbidity than older women. Although younger women may typically receive more aggressive treatment, this effect may be due to other possible explanations such as cancer being more disruptive to the lives of younger women, younger women having less experience coping with a chronic illness, and/or younger women having greater concerns about their future. This study examines factors that may explain age differences in the mental health of women who have been diagnosed with a first-time breast cancer. Analyses examined treatment and medical characteristics, as well as psychosocial factors such as coping strategies and the impact of cancer on life responsibilities.

Methods: This is an observational, longitudinal study of women aged ≥ 18 newly diagnosed with Stage I, II, or III breast cancer. Women were recruited through hospital clinics, community physicians, and advertisements and initially screened by chart review or telephone for eligibility. Eligible women completed a baseline survey within 8 months of diagnosis and 6, 12, and 18 months later. Women were recruited from Memorial Sloan-Kettering Cancer Center and the University of Texas-Southwestern University. The main outcome is the Mental Component Score (MCS) of the SF-35 Health Survey. Predictors included comorbidities, sociodemographics, coping strategies, and illness intrusiveness in different areas. Chart reviews provided data on stage, type of surgery, adjuvant therapy, and other medical and treatment factors. Linear mixed effects models were used to assess the effect of the various covariates on MCS across time.

Results to Date: Approximately 740 women were determined eligible on initial screening and sent baseline questionnaires; 658 of these surveys were returned (response rate 89%). Five women were subsequently determined ineligible because they were Stage IV ($N=1$), >8 months post diagnosis ($N=1$), or refused chart review ($N=3$). The final sample of 653 women falls into the following age categories: 18–44 ($N=125$); 45–54 ($N=206$), 55–64 ($N=167$); 65–74 ($N=102$), and 75+ ($N=43$). The majority were non-Hispanic White (89%) and married/partnered (72%); 52% were Stage I, 40% Stage II, and 8% Stage III. 72% received radiation therapy, 67% chemotherapy, 41% tamoxifen 41%, and 33% arimidex. Consistent with other studies, the MCS showed significant age effects with older women (over age 55) having better MCS scores ($p<.0001$) and these differences were maintained over the course of the study. Multivariable models including medical and psychological variables reduced the impact of age on MCS. Illness intrusiveness ($p<.0001$) and escapist coping ($p<.0001$) were the primary variables related to MCS over all time points. Tumor size was the only clinical variable significantly associated with MCS ($p=.004$). The effect of chemotherapy regimen varied somewhat over time ($p=.11$) with greater differences early in the follow-up period.

Conclusions: Illness intrusiveness and escapist coping explain some of the age differences in psychological morbidity. These results suggest that interventions targeted to reducing the impact of cancer on women's lives may reduce the psychological morbidity for younger women.

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P39-37: INTRUSIVE SYMPTOMS AMONG WOMEN WITH BREAST CANCER: THE ROLE OF CHILDHOOD ABUSE

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The severity of traumatic stress symptoms following the diagnosis of breast cancer is known to be higher among women who report previous stressful life events and inter-generational trauma, but research on the possible impact of childhood abuse is scant. A history of childhood abuse is generally quite prevalent among health care populations, and a substantial literature demonstrates the adverse effects of childhood abuse on adult physical and mental health. The present study investigated the effects of childhood abuse on intrusive and avoidant symptoms in women newly diagnosed with breast cancer. As part of a larger ongoing study, 330 patients from eight public and private hospitals were referred by their physicians and completed the Childhood Trauma Questionnaire (CTQ), Mental Health Inventory-18 (MHI), and the Impact of Events Scale - breast cancer (IES). Among the CTQ subscales, emotional abuse showed the strongest correlation with intrusion, with physical and sexual abuse showing lesser but significant correlations. Multivariate analysis, controlling for age, time since diagnosis,

other psychological symptoms, and other abuse subtypes, revealed that childhood emotional abuse was an independent predictor of breast cancer-related intrusive symptoms. These findings highlight the importance of additional research to explore links between childhood trauma and women's distress following breast cancer diagnosis and may suggest strategies for intervention. Because a cancer diagnosis may trigger negative cognitions and emotions that are consistent with prior trauma experiences, physicians and psychologists should consider previous life experiences that may affect patients' coping with cancer.

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P39-38: DEVELOPMENT OF A STRUCTURED PATHOLOGY TOOL FOR BREAST CANCER CARE AND RESEARCH

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Background: Pathology data is needed by every stakeholder involved in breast cancer care, reporting, and research including: clinicians, patients, imagers, insurers, researchers, trialists, registrars, industry, and others. However, data is acquired separately by each entity and each system has its own ontology and data quality standards. We believe that high quality data should be acquired in the course of routine care so that it is available to improve care and drive research.

Methods: We have reengineered the data collection process to ensure common, structured pathology data is collected once and available for use by all stakeholders. To create a functional tool that included the specific details of pathology and surgical staging, we engaged a multidisciplinary team to ensure all data elements required by each clinical and research team were included. Data elements required by the NCI and for registry reporting were also identified, and systems for electronic reporting reviewed. Phase 1 resulted in the development of an Access database, with a reporting tool used to conduct multidisciplinary surgical patient reviews. For Phase 2, we are building a caBIG compatible clinical, surgery and pathology staging tool on an open source platform that will create a common repository for pathology data which will provide access to quality data, create patient care reports and enable routine staging, rolling events into a summary of the surgical episodes. The accuracy and timeliness of registry reporting is being compared.

Results: The most comprehensive list of data is required by clinicians for high quality care. In Phase 1, the quality of registry data was 85% accurate for primary tumor data, but only 43% accurate for the complete list of procedures provided and receptor status. Cancer staging data is available from 6 months to 2 years after a cancer diagnosis. Clinical staging information is not routinely reported even when neoadjuvant treatment is initiated. In Phase 2, we are completing the development of a comprehensive web tool to enable complete pathology data for every surgical event. Clinical staging, and final surgical staging will be adjudicating and summarized by the surgeon, and will include all events that comprise the surgical episode. An extract of this data will be sent directly to the NCCC Registry (SEER Region 9) and to CIS-NET registry software. Secondary data entry will not be necessary. We anticipate that data can be sent to the registry 2 weeks after the final surgical procedure and that data accuracy will exceed 95%. Results will be reported spring 2008.

Conclusion: Pathology data is critical for breast cancer care and research and desired by all stakeholders. It is infinitely more efficient to enter the data correctly once, and use it many times, which is feasible using standard terminology and modern software platforms and caBIG compatible. If registry data is more accurate, registrars can enter data into the Phase 2 system rather than the current registry system, thus making data available for clinical care decisions, longitudinal, clinical, and translational research, quality control and improvement, outcomes tracking, and registry reporting with no

additional resources. Eventually, pathology data will be directly entered by pathologists (Phase 3), further increasing the efficiency of the system.

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P39-39: BLUEPRINT FOR REGIONAL EXCELLENCE IN BREAST CANCER CARE

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Background: We lack systems to integrate delivery of breast cancer with efforts to collect data for clinical and translational research, outcome tracking, quality improvement, registry reporting, and safety/morbidity monitoring. These efforts remain separate and disconnected. Electronic medical records will not unfortunately solve the problem because they are text based and lack the ability to sort and analyze data on patients to improve care and services and monitor the quality of care.

Methods: We approached the reengineering of data collection and distribution by meeting with all users of clinical data/stake holders (patients, clinicians, clinical, translational, and outcome researchers, insurers, genetic counselors, psychologists, registrars, social workers, and administrators) to determine what data they required. We defined the domains of how data on patients is being reused: clinical care reports, registry reporting, research studies and tumor banks, safety, quality and quality improvement, clinic process flow, and billing. We are working to harmonize data elements with standard definitions using common data elements (CDEs) to facilitate data exchange. We developed and tested a variety of web-based technology platforms that would enable capture, storage, and reuse of data. A cross-disciplinary development team worked to architect solutions.

Results: A significant portion of the necessary data (medical history, demographics, quality of life surveys, complications of care, outcomes) can be entered by patients themselves, along with their caregivers using web-based tools. Clinicians should focus on entering the specifics of diagnosis, staging, and treatments rendered and follow-up status. The data required for good clinical care encompasses almost all of the data needed for research, registry reporting, billing, etc. Thus what is required is an efficient way to store and reuse clinical data. After attempting to build and partner to create several technology solutions, we have now identified an open source data capture and storage tool, Tolven, which enables reuse of data.

We have reengineered patient services that leverage data capture and analysis: (1) co-ordinated diagnosis and evaluation; (2) survivorship; (3) personalized clinical trial matching; (4) clinical trial eligibility; and (5) decision services (visit preparation, automated sending of shared decision-making videos, and decision aids).

We are currently building a caBIG compatible clinical, surgery, and pathology staging tool for patient care management, and will be testing the ability to populate a decision aids, case report forms, and a research management system (Early Detection Research Network). We are also launching a patient-based national service for clinical trial matching (BreastCancerTrials.org).

Conclusions: A systems approach to clinical care delivery is possible and can integrate care, research, quality, and safety functions and fundamentally change care for women with breast cancer, enable us to monitor the quality of care, and accelerate the pace at which innovations are made broadly available.

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HBCU/MI PARTNERSHIP TRAINING AWARDS II

Poster Session P40

P40-1: CHARACTERIZATION OF TUMOR SUBTYPES IN AFRICAN AMERICAN AND LATINA BREAST CANCER PATIENTS IN SOUTH LOS ANGELES

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Background: Several studies have demonstrated that tumor tissue subtypes may influence the outcome of breast cancer patients. Those with basal-like tumors, with triple-negative phenotype have limited treatment options. Recent studies have shown that minority women, in particular, African American are more likely to express the triple-negative tumors compared to non-Hispanic Whites. Perhaps this difference may be one of the factors contributing to disparities in disease outcome and overall survival between different racial and ethnic groups. In our study, we have studied a cohort of African American and Latina women with equal socioeconomic status (SES) and access to care. The purpose of this study was to elucidate if these two ethnic groups with equal SES and access to care had differences in disease outcome and if this difference was related to tumor subtypes.

Methods: A total of 300 subjects were retrospectively selected. A total of 156 were African American and 144 Latina. Demographic, pathologic, and clinical follow-up information was collected from existing database. Subtype definitions were as follows: Luminal A (ER+ and/or PR+, HER2-); Luminal B (ER+ and/or PR+, HER2+); basal-like or triple-negative (ER-, PR-, HER2-); and HER2+/ER- (HER2+, ER-, PR-). The prevalence of breast cancer subtypes within racial and menopausal subsets was examined and the associations with tumor size, lymph node status, staging, and histologic grade were determined. Disease-free survival (DFS) was analyzed by Kaplan-Meier survival curves with log-rank test.

Results: The basal-like breast cancer subtype was more prevalent among premenopausal women. For instance, African American women (36%) compared to postmenopausal African Americans (28.1%), and premenopausal Latina women (31%), compared to postmenopausal Latinas (17%) (p=0.05). The HER2+/ER- subtype was also more prevalent among premenopausal Latina women (17%) than postmenopausal Latinas (14%). The rate in postmenopausal African American women was 9% and in premenopausal African American women 3% (p=0.046). Prevalence of Luminal A and Luminal B breast cancer subtypes were similar with race and menopausal status. Compared to Luminal A, basal-like tumors were more poorly differentiated (OR=6.4, p<0.001) while HER2+/ER- tumors were more associated with large tumor size (OR=3.1, p=0.01). The Luminal B tumors were also more likely to be associated with larger tumor size and were poorly differentiated (OR=3.0, p=0.002). Patients with basal-like and HER2+/ER- tumors had significant shorter DFS compared to patients with Luminal A tumors (log rank=11.97, p=0.0005, and log rank=8.95, p=0.002). The DFS was also reduced significantly in patients with Luminal B tumors than Luminal A tumors (log rank=7.09, p=0.007).

Conclusions: Our study suggests a highest prevalence of basal-like tumors (triple negative) in premenopausal African American women, followed by premenopausal Latina women. Premenopausal Latina women also had a higher prevalence of HER2+/ER- tumors. DFS was poor in both ethnic groups with either basal like tumors or those with HER2+/ER- tumors. These results suggest that tumor subtypes may contribute to the poor prognosis of young African American and Latina women with breast cancer.

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P40-2: Akt AND HER2 OVEREXPRESSION IN AFRICAN AMERICAN AND LATINA PATIENTS WITH BREAST CANCER

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Background: Breast cancer ranks second among cancer deaths in women. African Americans are at a higher risk to die from cancer than other ethnic groups. Breast cancer also is the most commonly diagnosed cancer and the leading cause of cancer death among Latina women. About 20% to 30% of breast cancer patients are diagnosed with an aggressive form of cancer that is associated with overexpression of the HER2 (HER2/neu) protein and its gene. Breast cancer patients with HER2 overexpression have poorer outcome. The biology of HER2 overexpression in breast tumors in African American and Latina women is poorly understood. The purpose of this study is to understand the clinical significance of activated Akt (phospho-Akt or pAkt) expression in breast tumors from African American and Latina patients with corresponding tissue HER2 overexpression. Cellular and molecular studies have shown that activation of the cell signaling PI3-kinase/Akt cascade via the HER2 and other receptor tyrosine kinases induce cell proliferation.

Methods: A total 234 African American and Latina patients were selected retrospectively. From this group, 141 tumor tissue samples were analyzed for tissue pAkt by

immunohistochemistry (IHC). This cohort consisted of 46 HER2 positive (3+ by IHC) and 95 HER2 negative tumors. The prognostic value of activated tissue Akt in relation to HER2 overexpression for disease-free survival (DFS) was determined by Kaplan-Meier survival curves with log-rank test. The relative risk (RR) of reducing disease-free survival was determined by Cox's proportional hazard regression with multivariate analyses.

Results: Patients with low pAkt and HER2 negative tumors had the best overall survival. As expected, HER2 overexpressing tumors with low pAkt had a decrease in DFS. Similarly, those with high pAkt and HER2 negative tumors had poor DFS. However, those with increase in both HER2 and pAkt had the worse DFS. Increase in pAkt was significantly associated with HER2 positive and lymph node positive breast tumors. Tumors with high HER2 and high pAkt were metastatic. Multivariate analysis demonstrated that in addition to the common risk factors such as larger tumor size, lymph node involvement, ER/PR negative tumors, and HER2 positive tumors, overexpression of pAkt was significantly associated with a decrease in 5-year DFS. Decrease in DFS with increase in pAkt was observed in both HER2 positive and negative groups. However, the DFS was similar between HER2-positive/pAkt-negative and HER2-negative/pAkt-positive groups.

Conclusions: Our data suggest that there may be differences in tumor phenotypes within the HER2 overexpressing breast cancer patients. The overexpression of pAkt may be a powerful prognostic marker for predicting DFS of breast cancer patients. Targeting pAkt for therapy in these high-risk populations may be an important opportunity to improve DFS of breast cancer.

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P40-3: TARGETED DCE-MRI FOR IMAGING AND CHARACTERIZATION OF SOLID TUMOR XENOGRAPHS

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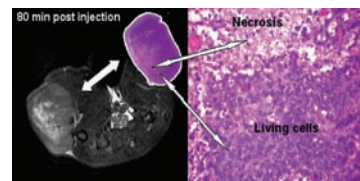
The advances in MRI imaging techniques have led to the increasing use of parametric images, which are designed to display physiological, pathological, and morphological features of tissue in one experiment. The dynamic contrast enhancement MRI (DCE-MRI) involves acquisition of a series of MR images before, during, and after an intravenous (IV) injection of a contrast agent (CA). After IV injection, the CA is distributed only in the extracellular space through passive diffusion. CA doesn't accumulate in normal cells, and it is rapidly excreted in the urine. The distribution of CA is generally nonspecific.

The development of liposomes tumor-specific targeting drug delivery has the potential for delivering imaging contrast agents more effectively than is currently achievable. A cell membrane receptor transferrin (TfR) is known to be overexpressed in 74% of breast carcinomas, 76% of lung adenocarcinomas, and 93% of lung squamous cell carcinomas. The expression level of TfR receptor is of great value in grading tumors and determining prognosis. There are two goals in this study: (1) to develop a liposome-Tf nanocomplex as MRI probe for systematic delivery of the conventional gadolinium-based CA to tumors and (2) to analyze the relationship between MRI dynamic contrast enhancement pattern and tumor pathology after administration of the newly developed probes with CA.

We have constructed liposome (Lip) nanoparticles modified with Tf on the surface as a ligand for specific targeting and CA (Magnevist) inside as the payload. These components together formed a liposomal nanocomplex, Tf-Lip-Mag, with a diameter less than 100 nm. In vitro analysis demonstrated that the Tf-Lip-Mag nanocomplex dramatically improved the uptake of CA in monolayer cultures of MDA-MB-231-luc human breast cancer and in PC-3-luc cells through both receptor- and Lip-mediated endocytosis. In vivo, the probe significantly enhanced the MRI signals and was superior to the use of clinical MRI CA alone. The DCE-MRI exhibits the heterogeneous signal enhancement by the liposomal nanocomplex probe and it correlates well with the pathology of the tumors (see figure).

We have demonstrated that our newly developed probes, Tf-Lip-Mag, increases the sensitivity and specificity of MRI. It also shows the ability of identifying the tumor pathological features based on the DCE-MRI pattern. The results indicate that the Tf-Lip-Mag nanocomplex has a potential for improving cancer detection and tumor characterization.

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Typical DCE-MRI correlated with the pathological features of the tumor

P40-4: TARGETED FLUORESCENT LIPOSOME NANOPARTICLES FOR MOLECULAR IMAGING OF BREAST CANCER XENOGRAPHS IN MOUSE

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Background and Objectives: Molecular imaging is developed on the basis of the concept that targeted delivery of contrast agents can specifically increase the detection sensitivity by targeting the difference in “molecular properties” between cancer and normal tissues. Transferrin receptor (TfR) is a cell-membrane internalizing receptor, which is overexpressed in various human malignancies including breast cancer. It is a potential target for targeted imaging. Herein, we developed a fluorescent liposome nanoparticles targeting TfR for optical imaging of human breast cancer xenografts in mice.

Methods: The targeted fluorescent liposome nanoparticles were constructed using near-infrared (NIR) fluorescent transferrin conjugate (Tf^{NIR}), NIR fluorescent dyes and fluorescently labeled (NBD) cationic liposomes (Lip^{NBD}). NIR dyes were first encapsulated in the Lip^{NBD} to yield NIR-loaded liposomes (Lip^{NBD}-dye). They were then linked to Tf^{NIR} to obtain targeted fluorescent liposome nanoparticles (Tf^{NIR}-Lip^{NBD}-dye). The binding and internalization of the nanoparticles were first analyzed in MDA-MB-231-luc breast cancer cells using confocal microscopy and flow cytometry. The imaging feasibility was then tested in solid tumor xenografts in nude mice using Xenogen optical imaging system.

Results: Confocal microscopy showed endocytosis of the fluorescent reporters from transferrin (Tf), liposome particles, and encapsulated dyes, separately, following incubation of the MDA-MB-231-luc cells with the nanoparticles. Pretreatment with Tf blocked the cellular uptake of the reporters indicating the importance and specificity of the Tf moiety for targeting. Quantification using flow cytometry revealed a 1.8-, 7.0- and 16-fold higher fluorescence intensity in cells incubated for 1 hour with Tf^{NIR}, Lip^{NBD}-dye, and Tf^{NIR}-Lip^{NBD}-dye, separately, than in control cells without probes (all $p < 0.05$). Systemic administration of Tf^{NIR} alone showed a preferential accumulation of the fluorescent signal in tumor xenografts produced in the lower back of nude mice. The fluorescent signal was clearly detectable at 10 min in tumors and reached the maximum intensity at 90-120 min after injection. The ratio of the signal from tumor to background from muscle ranged from 1.64 to 3.14, depending on the tumor sizes. Application of the Tf^{NIR}-Lip^{NBD}-dye nanoparticles further increased the signal from tumor to background ratio by up to 30% compared to Tf^{NIR} alone. Importantly, Tf^{NIR}-Lip^{NBD}-dye system is superior to Tf^{NIR} alone for imaging small tumors (<3 mm in diameter).

Conclusion: Our results indicate that TfR is a promising target for molecular imaging. Both Tf^{NIR} and Tf^{NIR}-Lip^{NBD}-dye are potentially useful for visualizing human breast cancer in clinic. Tf^{NIR}-Lip^{NBD}-dye is superior to Tf^{NIR} alone for imaging small tumors because of better delivery of fluorescent contrast agents to the tumors.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0291.

P40-5: SURFACE COATING AND BIOCONJUGATION OF QUANTUM DOTS FOR NON-INVASIVE DETECTION OF BREAST CANCER

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In literature, there are many reports on imaging cancer cells with quantum dots (QDs). However, optical imaging of live animals using QDs is still scarce. We have designed QDs conjugated with transferrin (Tf) as an optical imaging agent for breast cancer detection with high sensitivity and specificity. QDs are chosen to improve detection sensitivity due to their large extinction coefficient and emission of bright light in comparison to the organic fluorescent dyes. Transferrin was used as a ligand specifically targeting the overexpressed transferrin receptors on the MDA-MB-231 breast cancer cells.

The two steps carbodiimide chemistry process was used to obtain the QDs-transferrin conjugation. Carboxylated QDs were first activated by excess EDAC and sulfo-NHS at pH 6.5. Then the excess EDAC and sulfo-NHS are removed by Sephadex™. Later, the activated QDs were conjugated with transferrin at pH 8.3 and further purified by gel filtration. The successful linkage of transferrin to QDs was demonstrated by SDS-PAGE gel electrophoresis (Figure 1). In vitro uptake of QDs-Tf by MDA-MB-231 cells was confirmed by cellular labeling combined with flow cytometry (Figure 2), optical imaging, and fluorescent microscopy techniques. For live animal imaging, novel multi-dentate ligands will be used for surface coating to improve the stability and biocompatibility of QDs under harsh in vivo conditions.

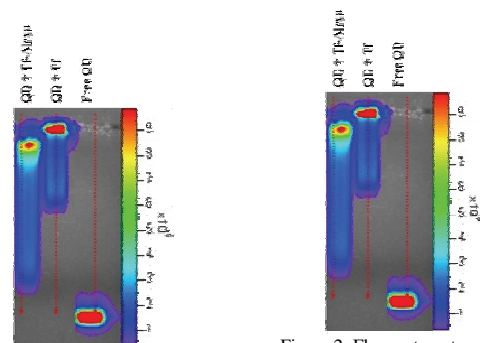


Figure 1: SDS-PAGE of quantum dot (QD) and QD-Transferrin conjugates

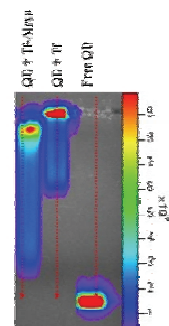


Figure 2: Flow cytometry results of MDA-MB-231 breast cancer cells with and without QD-Tf conjugates labeling

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P40-6: OPTICAL IMAGING OF AN EX VIVO MODEL CANCEROUS HUMAN BREAST USING INDEPENDENT COMPONENT ANALYSIS

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Background and Objectives: The interest in developing optical mammography derives from a need for safe, noninvasive, affordable modalities for detection and diagnosis of tumors in breast at early stages of growth when those are more amenable to treatment. The scattering and attenuation of light by breast tissues limit the resolution and accuracy of three-dimensional (3D) location estimation of a breast tumor. We are developing an approach for optical imaging using independent component analysis (OPTICA) for improving the accuracy of target localization and enhancing the spatial resolution. In this paper, we use OPTICA to image and locate a tumor and other structures embedded in a realistic breast model assembled using ex vivo human breast tissues, as a prelude to in vivo breast imaging.

Brief Description of Methodologies: OPTICA uses a multi-source target probing and multi-detector signal acquisition scheme to obtain multiple spatial and angular views of the sample for detecting and locating the target(s). The model breast was a 70 mm x 55 mm x 33 mm slab composed of ex vivo female human breast tissues provided by National Disease Research Interchange under an Internal Review Board approval at the City College of New York. A tumor (infiltrating ductal carcinoma) with small amount of normal tissues in the margins with an overall dimension of 8 mm x 5 mm x 3 mm was embedded in the mid-plane of the slab. A 200 mm diameter optical fiber delivered a 784 nm, 300 mW continuous wave diode-laser beam that was collimated to a 1 mm spot for sample illumination. The sample was step scanned in an x-y array of grid points to realize the multi-source scanning. For illumination of every grid point in the input plane of the sample, a 1024 x 1024 pixels charge coupled device camera recorded a two-dimensional image of the exit plane illuminated by the transmitted diffuse light. Independent component analysis of these images provided target location. A back-projection technique provided estimate of the target cross section.

Results: The location of the tumor was determined to within ~1 mm in all three dimensions. In addition, two pieces of previously unknown fibro-glandular tissues were identified and their location was estimated. Subsequent histological analysis affirmed the presence of the two fibro-glandular pieces. The estimate of cross section of the tumor was accurate to within 20% of the actual size in the lateral dimensions.

Conclusion: Results of this experiment demonstrate that OPTICA is potentially suited for detection of breast tumors at early stages of growth and need to be tested in a detailed in vivo study.

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P40-7: BIOLUMINESCENCE IMAGING FOR MONITORING THE RESPONSE OF A LUCIFERASE TRANSFECTED HUMAN BREAST CANCER CELL LINE SUBJECTED TO HYPERTHERMIA

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This study was designed to assess the feasibility of using a bioluminescence assay for rapid estimate of cell death in luciferase transfected MDA-MB-231-luc-D3-H1 human breast cancer cells subjected to 43°C hyperthermia. Colony formation assay, which takes 7 to 15 days, is the gold standard for in vitro evaluation of cell survival after expo-

sure to radiation, hyperthermia or cancer chemotherapeutic agents. More rapid assays that yield results that are closely related to clonogenic survival are needed. MDA-MB-231-luc-D3H1 is a stable luciferase transfected breast cancer cell line, which lends itself to bioluminescent detection and imaging in the presence of D-luciferin. Three different assays of cell viability were used to study the response of MDA-MB 231-luc-D3H1 human breast cancer cells to 43°C hyperthermia treatment, which consisted of immersing sealed 96-well plates containing 10^4 cells per well, in a temperature-controlled water bath. The relative survival values determined by the different assays differed considerably. Immediate assay after 2 hours at 43°C indicated relative survival values of 30%, 68%, and 2% based on clonogenicity, MTT assay, and luciferin-based luminometry, respectively. When the MTT assay was delayed, to allow cells to proliferate for 5 days post-hyperthermia, the relative cell survival was 34%. The delayed MTT assay, however, covers only a narrow range of cell survival and fails to detect low levels of toxicity. After hyperthermia treatment, MDA-MB231-Luc human breast cancer cells were treated with D-luciferin (150 µg/ml) for luminometric measurement of bioluminescence using a Xenogen IVIS 200 imaging system. The luminescence was related to the number of viable cells over a wide range (10^2 to 10^5 cells per well). The decrease in luminescence was detectable immediately after heat treatment. This decrease was related to the duration of heating at 43°C. When compared to clonogenicity measurements, the luminometric assay overestimated the effect of hyperthermia. In conclusion, relative to the gold standard of clonogenic assays, MTT assay overestimates viability and covers only narrow range of cytotoxicity while bioluminescence underestimates viability of cells subjected to hyperthermia. Luminometric assay of response to hyperthermia is rapid in bioluminescent cells but is not closely related to the clonogenic potential. This suggests that other ATP-based assays of cell viability will also overestimate cytotoxicity when compared to clonogenicity assays. Nonetheless luminometric estimate of ATP in cells can be used for rapid screening of cytotoxic regimens. Caution has to be exercised while interpreting cell viability results based on different assays.

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P40-8: PSYCHOSOCIAL CORRELATES OF MAMMOGRAPHY SCREENING IN OLDER AFRICAN AMERICAN WOMEN

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Purpose: The purpose of this pilot study was to examine psychosocial predictors of older low-income African American women's adherence to mammography screening; predictors included cancer fatalism, health beliefs, optimism, and social support as well as demographic and clinical characteristics. Secondary aims included testing the efficacy of the Health Belief Model in predicting mammography screening.

Methods: A cross-sectional survey design was used. African American women aged 50 and older living in low-income housing completed questionnaires that included demographic information, breast cancer screening behavior, knowledge of breast cancer screening guidelines, Powe Fatalism Inventory, Champion's Revised Susceptibility, Benefits, and Barriers Scale for Mammography Screening, Life Orientation Test, social support items adapted from the Medical Outcomes Study Social Support Scale, and perceptions of general health from the SF-36. Logistic regression was used to assess the effect of cancer fatalism on recent mammography screening controlling for demographic factors, optimism, social support and self-rated health. Multivariable regression was used to explore predictors of the Health Belief Model.

Results: The 198 participants were an average of 67 years old; most had a high school education or less (72%) and were not married (91%). Approximately half (50%) reported they had had a mammogram within the last year. Women who believed they were susceptible to breast cancer ($p=0.0243$), had fewer perceived barriers to getting a mammogram ($p=0.0106$), and more functional and emotional social support ($p=0.0110$) were more likely to have had a mammogram in the last year. Age, education, marital status, self-rated health, and having a family member or friend diagnosed with breast cancer did not predict mammography screening in the past year. Women who were less educated and who had higher levels of cancer fatalism had higher levels of perceived susceptibility to breast cancer and more perceived barriers to mammography screening. Women with lower levels of dispositional optimism perceived more barriers.

Conclusions: Women were more likely to have had a mammogram within the past year if they believed they were susceptible to breast cancer, had fewer barriers to obtaining a mammogram, and higher levels of functional and social support regarding their general and health.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0458 and Piedmont Alliance for Cancer Research and Education.

P40-9: A COMPREHENSIVE VALIDATION ANALYSIS FOR DIGITAL MAMMOGRAPHY SEGMENTATION ALGORITHMS

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Howard University

In an effort to improve state-of-the-art computer-aided diagnoses (CAD) for cancer, a comprehensive validation analysis is presented. The performance of three existing digital mammography segmentation algorithms were tested and evaluated against manual segmentation results produced by two expert radiologists. This is an improvement of an early methodology used for the evaluation of boundary algorithms on medical images. The mammography images used were acquired from the Digital Database for Screening Mammography (DDSM) and subsequently used as ground truth. We assessed three existing segmentation algorithms: (a) Region Growing Combined with Maximum Likelihood (ML) Model, (b) Gradient Vector Flow (GVF) Model, and (c) the Standard Potential Field (STD) Model. We applied a comprehensive statistical metric. We concluded that the ML Model yielded not only the best accuracy, specificity, percent error, and algorithm ranking, but also the greatest ratio of average computer-to-observer agreement and average inter-observer agreement (WT'). The upper limit of the 95% Confidence Interval (CI) was also found to be greater than 1.0, thus each individual observer is a reliable member of the group.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0267.

P40-10: PRELIMINARY STUDY ON NORMAL MAMMOGRAM CHARACTERIZATION

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In this paper, we are introducing a recent idea that just came up a few years ago, which is of characterizing and identifying the features and patterns of normal tissues instead of the abnormal ones. Most studies [7-12] are done on identifying the abnormalities in mammograms and only little work was done on the normal mammograms. So, we aim to focus on normal mammograms. Other studies [16] found that the relative risk of developing breast cancer in women with dense breasts is 400% higher than in women with fatty, non-dense breast tissue. Hence we will focus our study on the "hard to classify" dense breast cases. Most studies are done on the mammograms regardless of the tissue type (dense/fatty) and therefore, affect the quality of any classifier used. In this study we need to introduce the idea of a CAD that will be capable of identifying the normal tissue by extracting mammo-specific features, and using a classifier to recognize the normal patterns of dense breasts. The main aim is to reduce the false negative rate significantly. Only few preliminary experiments are done to extract some features and classification is then performed using SVM. The quality of the system when each feature is used shows that different features give better results with some kernels than the others. Intensive comparative will be done later to explore the optimum set of features and the corresponding best kernel that will significantly improve the performance of the whole system and give min false rate. The main ideas described here are as follows:

1. Extracting and characterizing the features and patterns of normal mammograms instead of abnormal.
2. More specific, we will be working on the dense breasts only, since they are the "hard to classify" cases and most errors of misinterpretation happen on dense breasts.
3. Extracting mammo-specific features that suit mammograms and more specifically, dense breasts. Only existing features were tested in the preliminary results.
4. Applying for the first time to the best of my knowledge the Min Redundancy Max relevance feature selection that will be used to select the most relevant features to dense -normal mammograms.
5. SVM will be used as a weak classifier and its performance will be enhanced using Adaboosting technique.

In this paper, we only introduced the idea and did some preliminary results on some existing features and compared their quality when each feature is used individually with the classifier (i.e., SVM). We also examined the effect of different kernels used with the features but no consistent results came up. We concluded that still more intensive research needs to be done to investigate the best set of features that can well-identify the patterns of normal mammograms.

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TRANSLATIONAL RESEARCH AWARDS II

Poster Session P41

P41-1: VEGI, AN ANTIANGIOGENIC CYTOKINE, STIMULATES DENDRITIC CELL MATURATION

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Angiogenesis is an essential component of chronic inflammation that is linked to carcinogenesis. We found that human vascular endothelial growth inhibitor (VEGI, TNFSF15), an endothelial cell-produced antiangiogenic cytokine, is able to induce mouse dendritic cell (DC) maturation, a critical event in inflammation-initiated immunity. VEGI-stimulated bone marrow-derived immature DCs display early activation of maturation signaling molecules NF-kappa-B, STAT3, p38 and JNK, and cytoskeleton reorganization and dendrite formation. Death-domain containing receptor-3 (DR3) is at least partially responsible for the transmission of VEGI signals. Additionally, the cells reveal significantly enhanced expression of mature DC specific marker CD83, secondary lymphoid-tissue directing chemokine receptor CCR7, the major histocompatibility complex class-II protein (MHC-II), and co-stimulatory molecules CD40, CD80, and CD86. Functionally, the cells exhibit decreased antigen endocytosis, increased cell-surface distribution of MHC-II, and increased secretion of interleukin-12 and TNF. Moreover, VEGI-stimulated DCs are able to facilitate the proliferation and differentiation of CD4+ naive T cells in co-cultures. These findings suggest that the anticancer activity of VEGI arises from coupling the inhibition of endothelial cell growth with the promotion of the adaptive immune mechanisms through the stimulation of DC maturation.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0250.

P41-2: A LONG JOURNEY FROM THE LABORATORY DISCOVERY TO CLINICAL TRIALS: TALE OF ENDOGLIN, A MARKER OF TUMOR VASCULATURE

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The major objective of the present study is to develop novel vascular targeting anti-angiogenic agents to treat breast and other cancers. Recently FDA approved our IND (Investigational New Drug) application of a novel agent, termed c-SN6j or TRC105, for clinical trials; the IND application was helped by Traccon Pharma (San Diego, CA). c-SN6j is a chimerized (humanized) monoclonal antibody (mAb) defining endoglin (EDG; CD105). EDG that is a homodimeric cell membrane antigen on leukemia and endothelial cells was discovered by us (Haruta and Seon, 1986) and Letarte's group (1988). EDG is a proliferation-associated antigen and is strongly expressed in angiogenic vasculature of human tumors. In addition, EDG is essential for angiogenesis and an ancillary TGF- β receptor.

Initially we used an anti-EDG mAb and its drug conjugates to treat human leukemia in animal models. More recently, however, we have been using anti-EDG mAbs and their immunoconjugates (immunotoxins and radioimmunoconjugates) to target tumor vasculature in several animal models. We generated 12 anti-EDG mAbs that show strong reaction with tumor vasculature but little reaction with tumor cells per se when tissues of various human solid tumors were tested by immunohistochemical staining. We selected four (including SN6j) of these for animal model studies. Importantly, these mAbs and/or their immunoconjugates showed both anti-angiogenic activity and vascular targeting activity. In animal studies, systemic administration of selected anti-EDG mAbs or their immunoconjugates could suppress angiogenesis, tumor growth, and metastasis without overt toxicity; the tumors included both established and new tumors. The observed efficacy of these mAbs in mice is remarkable in view of the fact that these mAbs only weakly cross-react with murine endothelial cells. These mAbs were much more effective for targeting human vasculature than targeting murine vasculature in human skin/SCID mouse chimeras in which human tumors were established in human skin grafts in SCID mice. The results suggest that these mAbs will be much more effective for cancer therapy in humans than in mice. Combination of SN6j with cyclophosphamide or doxorubicin showed synergistic antitumor efficacy in animals.

We have been performing several tests to understand the mechanisms by which naked (unconjugated) anti-EDG mAbs and c-SN6j suppress angiogenesis, metastasis, and tumor growth. The results indicate that the suppressive activities involve multiple mechanisms.

To facilitate clinical application, we generated a recombinant human/mouse chimeric mAb termed c-SN6j from an anti-EDG mAb SN6j. A dose escalation study of c-SN6j was performed in nonhuman primates by repeated iv injections to evaluate potential toxicity, immunogenicity, and pharmacokinetic parameters. The results are very encouraging. No significant toxicity was detected in the primates by several criteria. Immune response to the mouse part of c-SN6j was minimal in most animals. A large volume of data from the in vitro and in vivo studies of SN6j and c-SN6j was presented to FDA to obtain approval of the IND application of c-SN6j. cGMP-compliant c-SN6j

was produced for clinical trials with the help of Traccon Pharma. We should have some early results of Phase 1 clinical trials of c-SN6j (TRC105) when the Era of Hope 2008 Meeting is held.

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P41-3: BREAST DENSITY MEASURED BY FOUR DIFFERENT METHODS IS INDEPENDENTLY PREDICTED BY BODY FAT COMPOSITION AND DISTRIBUTION, HISPANIC ETHNICITY, NUMBER OF PREGNANCIES, AND INDICES OF LIVER FUNCTION

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University of Texas Medical Branch, Galveston

Women with very high breast density (BD), which reflects amounts of glandular breast tissue, have a 4 to 6-fold greater breast cancer risk compared to women with little or no dense breast tissue. BD is a surrogate marker for assessing effects of interventions on breast cancer risk, and is most commonly measured by a laborious, subjective histogram segmentation method (HSM). We discovered serendipitously during initial data mining in a study of normal women that information routinely recorded in mammogram reports of the full field digital mammographic unit, such as the radiation dose absorbed ('Raddose') and radiological thickness ('Radthick'), correlated with BD of each imaged breast. We developed a multiple regression model to automatically compute BD (%-BD_{stat}) upon the completion of image acquisition omitting the HSM (%-BD_{HSM}) step. The statistical model is: %-BD_{stat}=634.9 -0.0067*Pre-exposure Dose +1.5298*Pre-exposure Breast Compression Thickness -0.1752*RadDose +6.3329*Pre-exposure KVP -0.056*Anatomical Mean Intensity -0.0103*Segmentation Threshold -2.4577*Post-exposure Breast Compression Thickness -0.0649*Compression Force -4.559*Detector Sensitivity -1.758*Filter +37.904*Anode (Lu et al *Phys. Med. Bio.* 52 (2007) 4905, <http://stacks.iop.org/0031-9155/52/4905>). We then determined whether BD assessed by Raddose, Radthick, %-BD_{stat}, and %-BD_{HSM} was predicted by body composition measurements, fasting blood levels of proteins made in liver such as sex hormone binding globulin (SHBG), IGFI, IGFII, and C-reactive protein (CRP), several indices of liver function [alkaline phosphatase (AP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST)] and lipids (HDL, LDL, triglycerides etc), insulin, estradiol, progesterone, multiple anthropometric variables, and the number of completed pregnancies. Strong correlations were found by Pearson correlation analyses with Raddose, Radthick, %-BD_{stat}, and %-BD_{HSM}. Independent predictors for Raddose, Radthick, %-BD_{stat}, and %-BD_{HSM} were strikingly similar in several multivariate analysis models. In model 1, which included several liver function tests and lipid profiles, independent predictors were HDL, AP, ALT and AST, which explained 10% to 19% of the variance in BD. Model 2 included SHBG, CRP, IGFI, IGFII, insulin, estradiol, and progesterone, and revealed that SHBG, CRP, estradiol, and IGFs are independent predictors explaining up to 18% of the BD variance. Model 3 included all anthropometric variables, and showed %-body mass as fat, amount of body fat, and waist circumference explained >30% of the variance. In model 4, which included the independent predictors from models 1-3 plus additional variables, the most consistent independent predictors were %-body fat, waist circumference, Hispanic ethnicity, number of completed pregnancies, and levels of several liver-derived proteins such as SHBG, CRP, IGFs, HDL, ALT, AST, or AP, explaining >40% of the BD variance. The similarities in the strong predictive factors for the various BD measures suggest that Raddose and Radthick provided in the digital mammogram reports and %-BD_{stat} predicted from our regression model are suitable and convenient alternatives to the HSM for estimating %-BD. This approach can greatly facilitate research on breast cancer risk. Moreover, liver alterations in healthy women may influence breast cancer risk.

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P41-4: RESIDUAL CANCER BURDEN AFTER NEOADJUVANT TAXANE-ANTHRACYCLINE CHEMOTHERAPY IS INDEPENDENTLY PROGNOSTIC IN EVERY PHENOTYPIC SUBSET OF BREAST CANCER

W. Fraser Symmans, Florentia Peintinger, Christos Hatzis, Rebekah Hubbard, Henry M. Kuerer, Vicente Valero, Aman U. Buzdar, Gabriel N. Hortobagyi, and Lajos Pusztai

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Background: Support from the DOD BCRP enabled the creation of a new method to measure the extent of residual cancer burden (RCB) after neoadjuvant chemotherapy (*J. Clin. Oncol.* 2007;25:4414-22). The aims of this study were to evaluate the prognostic strength of RCB measurements according to tumor phenotype in patients who were uniformly treated with neoadjuvant chemotherapy, and to determine the reproducibility and prognostic strength of RCB measurements from four pathologists.

Methods: We studied 559 patients who received paclitaxel followed by 5-fluorouracil, doxorubicin, and cyclophosphamide (T/FAC) as neoadjuvant chemotherapy, including 241 previously reported and 318 new cases. No patients received any trastuzumab therapy. RCB index was determined from retrospective pathologic review of the surgical resection specimen (www.mdanderson.org/breastcancer_RCB). Tumor phenotype was determined by standard tests for HER2 status, then estrogen receptor (ER) status, and categorized as: HER2+, ER+/HER2-, or ER-/HER2-. We selected from the original cohort 100 cases that contained residual in situ, invasive, or metastatic carcinoma, to be evaluated by four pathologists. Association of RCB with distant relapse-free survival (DRFS) was evaluated using Kaplan-Meier (log-rank test) and Cox regression analyses.

Results: RCB was significantly associated with DRFS in both the original and new cohorts (median follow-up 79 months and 31 months, respectively), and in each phenotypic group (Table 1). DRFS was identical for RCB-0 and RCB-I. RCB was significantly prognostic (HR 2.08, 95% CI 1.74-2.52, $P < 0.0001$) in multivariate analysis after adjusting for phenotype ($P = 0.0004$), age dichotomized at 50 ($P = 0.03$), clinical Stage at diagnosis ($P = 0.11$), and use of adjuvant hormonal therapy ($P = 0.08$).

Table 1				
	Number	RCB Index (continuous) HR (95% CI)	P value	RCB Classes (0-III) Log-rank test (P value)
Original cohort	241	1.82 (1.42-2.39)	<0.0001	<0.0001
New cohort	318	1.74 (1.40-2.19)	<0.0001	<0.0001
HER2+	142 (25%)	1.86 (1.45-2.41)	<0.0001	<0.0001
ER+/HER2-	256 (46%)	1.80 (1.26-2.66)	0.0009	0.002
ER-/HER2-	161 (29%)	2.35 (1.80-3.14)	<0.0001	<0.0001

The overall concordance correlation coefficient for the continuous RCB index among the four pathologists was 94.4%, with replicate assessments having excellent accuracy (99.4%) and good precision (95%). For each observer, RCB index was prognostic for distant relapse-free survival (66 months median follow up): HR 3.46 95% CI 2.07 to 6.23, HR 2.84 (1.78 to 4.84), HR 2.78 (1.77 to 4.59), and HR 2.66 (1.68 to 4.49).

Conclusions: Residual cancer burden after neoadjuvant T/FAC chemotherapy is strongly prognostic in all phenotypic subsets of breast cancer, and can be reproducibly assessed by different pathologists.

Impact: Residual cancer burden is being included as a response endpoint in current neoadjuvant clinical trials and to develop predictive biomarkers. These new results add to the clinical confidence in this measurement of response to chemotherapy.

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P41-5: FLUORODEOXYCYTIDINE INHIBITS DNA METHYLATION AND INDUCES EXPRESSION OF TWIST1 IN THE HUMAN BREAST CANCER CELL LINE MDA-MB 231

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Background: Hypermethylation of cytosine-guanine dinucleotides (CpG) in the 5'-regulatory region is a frequent mechanism of inappropriate silencing of tumor suppressor genes. In breast cancer and other cancers, hypermethylation may contribute to the neoplastic character and resistance to chemotherapeutic agents. 5-fluoro-2'-deoxycytidine (FdCyd), as a chemically stable DNA methyltransferase inhibitor, is being investigated in our laboratory for its ability to inhibit DNA methylation and to induce the re-expression of genes silenced by hypermethylation in human breast cancer. *Twist1* is silenced by hypermethylation of a 5'-region rich in CpGs (a CpG "island") in the human breast cancer cell line MDA-MB 231. Although its relationship to tumorigenesis is not clear, the DNA methylation and messenger RNA (mRNA) expression of *Twist1* may serve as a marker for the biological activity of inhibitors of DNA methyltransferase.

Methodologies: Treatment of DNA with bisulfite converts unmethylated cytosines to uracils but does not convert methylated cytosines. Amplification of the bisulfite-treated DNA by polymerase chain reaction (PCR) allows the initial methylation status to be read as differences in the sequence of the amplified product. For bisulfite sequencing, clones of individual DNA molecules are sequenced. For methylation-specific PCR (MSP), primers are designed so that only methylated or unmethylated sequences are amplified. To determine the mRNA expression, the RNA is first converted to DNA and then amplified by PCR (RT-PCR).

Results: Analysis of DNA methylation by both MSP and bisulfite sequencing demonstrated decreased methylation in the 5' CpG island of *Twist1* after treatment of MDA-MB 231 cells with FdCyd. The overall frequency of unmethylated CpG dinucleotides in the amplified sequence before FdCyd treatment was $8.1 \pm 3.3\%$ ($n=10$). After treatment with 1, 10, or 100 μM FdCyd for 4 weeks, the overall frequency of unmethylated CpG dinucleotides increased to $20.1 \pm 8.1\%$ ($n=8$), $18.9 \pm 8.5\%$ ($n=10$), or $18.1 \pm 6.0\%$ ($n=10$), respectively. However, the unmethylated percentage at individual CpG dinucleotides after FdCyd treatment ranged from 0 to 87.5%. To determine if the decrease

in DNA methylation led to an increase in mRNA expression, *Twist1* mRNA was quantified by real-time RT-PCR. We found that the mRNA level of *Twist1* in MDA-MB 231 cells increased after treatment with FdCyd in a time- and concentration-dependent manner. Expression was induced 2.1-, 3.2-, or 9.6-fold compared to untreated cells by 1, 10, or 100 μM FdCyd treatment for 4 weeks, respectively.

Conclusion: These data indicate that FdCyd inhibited DNA methylation and induced *Twist1* gene expression in the MDA-MB 231 human breast cancer cell line.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0538.

P41-6: REPRODUCIBILITY OF GENOME-SCALE CHEMOSENSITIVITY SIGNATURES ON BIOLOGIC REPLICATE BREAST CANCER SAMPLES

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Background and Objectives: Genome-scale expression profiling has now been extensively used for research on breast cancer biology and has led to new insights regarding disease heterogeneity. However, clinical application of this technology has been limited by technical considerations, such as the need for fresh-frozen tumor samples for good RNA quality and concerns about reproducibility of the expression data obtained. Using genome-scale expression data derived from cancer cell lines and tumor biopsies, we have developed expression signatures for predicting response to specific cytotoxic chemotherapies. These signatures will be assessed in a soon-to-open preoperative breast cancer treatment trial. Our objective was to assess the reproducibility of the chemosensitivity predictions from expression profiling in a series of breast cancers.

Methods: Fourteen individual patients with multiple frozen cores were identified in the Duke Breast SPOR tissue repository. These multiple separate cores were assessed for percent invasive cancer cellularity, primary tumor size, and standard biomarker assessments. RNA was extracted, processed, and hybridized to Affymetrix H133 Plus 2.0 microarrays. This provided a mechanism to examine multiple biologic replicates for each patient. Gene expression based signatures were generated from post-processed data for sensitivity to doxorubicin and docetaxel, as derived and validated in retrospective patient studies (Potti et al. *Nat. Med.* 2006). For each signature, tumor samples with a posterior probability of response $>60\%$ were classified as "sensitive," according to the cut-offs for treatment assignment in the prospective trial design.

Results: One sample was run in quadruplicate, eight samples in triplicate, and five in doublet replicates. In an overall assignment of most sensitive to doxorubicin, most sensitive to docetaxel, or resistant to both agents, complete concordance was observed in 9 of 14 cases. The doxorubicin signature was concordant in 9 of 14 cases, while the docetaxel was concordant in 11 of 14 cases. When analyzed on a continuous scale, the intra-class correlation coefficients for the doxorubicin and docetaxel signatures were 86% and 90%, respectively. Notably, invasive cancer cellularity ranged from 10% to 90%, and did not impact appreciably on signature robustness.

Conclusions: When applied to replicate frozen core samples from a single case, chemosensitivity signatures appear robust and reproducible. Since the cores analyzed were biologic replicates, discordant cases may represent true heterogeneity within cancers from the individual patients. The described methods will be used for analyzing samples in the prospective clinical trial.

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P41-7: MEMBERS OF A GENE FAMILY OF SMALL INTEGRIN BINDING PROTEINS ARE INFORMATIVE SERUM MARKERS FOR DIFFERENT PHASES OF BREAST CANCER PROGRESSION

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Tumor progression involves modulation of cell adhesion, proliferation, immune evasion, apoptosis, angiogenesis as well as migration and metastasis. We have been studying a gene family of five identically oriented tandem genes within a 375,000 bp region on chromosome 4. This family, SIBLINGs (for Small Integrin Binding Ligand N-linked Glycoproteins), includes bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), osteopontin (OPN) and matrix extracellular phosphoglycoprotein (MEPE). Elevated expression of BSP and OPN has been reported in a number of different cancers. The goal of our research was to investigate the biology and biomarker utility of all five SIBLINGs in breast cancer.

Tissue biopsies and sera from subjects were obtained under IRB approved protocols. Study subjects included women who were disease-free and women with breast cancer.

A set of 52 normal and breast cancer biopsies were analyzed for SIBLING expression levels by microarray analysis. Competitive ELISAs were developed to measure BSP, DMP1, DSPP, OPN and MEPE in serum from 115 normal women and women with breast cancer. The presence of specific SIBLINGs in serum was confirmed by western blot analysis using monoclonal antibodies directed against each SIBLING. These same monoclonal antibodies were used for immunohistochemical analysis of SIBLING protein levels in biopsies. Markers of immune activation, inflammation and apoptosis were also assessed in serum and biopsies. Mean values between stages were compared by ANOVA and correlations between markers were tested by Pearson correlation.

Our research has shown that SIBLINGs are elevated to different degrees in serum from patients with breast cancers. BSP and OPN exhibit significant correlation with tumor stage while elevated DSPP levels were associated with late stage cancer and all three were associated with poor survival. DMP1 levels decreased with advancing tumor stage and MEPE levels in serum were actually reduced in breast cancer sera. SIBLINGs exhibit different gene expression levels in different cancer types (e.g., lobular versus ductal). BSP, DMP1 and OPN were found to bind and modulate the activity of specific matrix metalloproteinases (MMPs) and exhibited correlated expression in cancer with their specific MMP binding partners. OPN levels in serum and biopsies were associated with markers of immune activation across all disease stages, while DMP1 levels were associated with markers of inflammation in early stages and markers of apoptosis in later stages.

Women with breast cancer have a pattern of serum SIBLING levels that provide a "fingerprint" that reflects disease biology, severity and outcome. SIBLINGs have the capacity to influence the tumor microenvironment through MMP-dependent degradation of the extracellular matrix, release of growth factors as well as angiogenic and apoptotic factors, thereby altering survival, proliferation, migration, and metastasis. The association of OPN with immune activation is consistent with OPN production by macrophages and macrophage infiltration in tumor progression. DMP1 modulation of MMP-9 activity may contribute to chronic inflammation and early neoplastic transformation, while at later stages it may contribute to increased apoptosis. The distinct patterns of SIBLING protein levels in serum and biopsies are consistent with these proteins acting at multiple distinct stages in tumor progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0684 and National Institutes of Health.

P41-8: CIRCULATING CA27.29 AND MAMMAGLOBIN IDENTIFY METASTASES PRIOR TO CLINICAL RECURRENCE IN A PROSPECTIVE STUDY

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Clinical assays to detect circulating MUC-1 (CA27.29) and CEA are well characterized and in frequent use; however, there are few prospective studies to evaluate if the markers have prognostic value, are clearly predictive of worse outcome, or can be reliably detected before recurrence. Mammaglobin (MGB) is a breast-specific protein that is upregulated in breast tumors and a molecular marker of metastases but has not been extensively assessed as a circulating tumor marker.

Objective: To evaluate CA27.29, CEA, and MGB in consecutive plasma samples from patients enrolled in a prospective clinical trial.

Methods: Study patients (n=275) with invasive breast cancer (Stage I-III) were a cohort of the prospective ECU/AAMC sentinel node trial. Blood draws were scheduled quarterly for the first year postsurgery, every 6 months for the next 2 years, and then annually. Plasma samples (n=1,510) were tested by immunoassay at a clinical laboratory for CA27.29 and CEA. MGB was analyzed on a 63-patient subset (n=234) by conventional sandwich ELISA or bead-based technology. Investigators were blinded to patient identity; results were not provided to physicians. Only patients with samples available for testing within 2 years of recurrence were analyzed by log rank and Cox PH. Factors tested in multivariate analysis were tumor size, nodal status, histologic grade, and ER/PR status.

Results: Markers were elevated in at least one plasma sample >3months after surgery in 10% (MGB), 16% (CEA), and 17% (CA27.29) patients tested. Elevated CA27.29 correlated with tumor size and positive nodes. Neither MGB nor CEA correlated with clinicopathologic factors. At a median follow-up of 4 years, 15 patients have recurred

with distant metastases, and samples tested for CA27.29 and CEA. Only CA27.29 identified distant metastases prior to recurrence in a significant number of patients and was associated with decreased disease-free survival (6 patients; p<0.02 by log rank analysis). The interval between elevated CA27.29 and recurrence ranged from 13-436 days (median=324). Nodal status was the only other factor predictive of distant recurrence by log rank (p=0.04; HR 2.5; 1.0-6.1). When CA27.29 status was entered into the Cox model as a pair with other factors, CA27.29 was the only significant predictive variable (p=0.04; HR 3.0; 1.0-8.6 when tested with nodal status). In the patient subset analyzed for MGB, elevated MGB was elevated 210 and 291 days before distant metastases in 2 of 4 recurrent patients (Chi² p<0.01). One of these patients had consistently normal levels of CEA and CA27.29; the other had elevated CA27.29. Early analysis suggests that MGB velocity (ng/mL/month) may be a more powerful indicator of outcome.

Conclusions: This prospective blinded study indicates that CA27.29 is a valuable tumor marker with clinical utility and an independent predictor of distant metastases and decreased disease-free survival. MGB holds significant promise as a predictive circulating marker and merits further study. Both markers have detected occult disease many months prior to clinical recurrence when there may be opportunity for effective clinical intervention. Serologic biomarkers warrant continued evaluation as a noninvasive means of surveillance to detect subclinical metastases, guide treatment, and potentially improve outcome.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0239.

P41-9: GENOMIC EXPRESSION PROFILES TO DIRECT THE USE OF PREOPERATIVE CHEMOTHERAPY FOR EARLY-STAGE BREAST CANCER: PROTOCOL AND INFRASTRUCTURE DEVELOPMENT

Paul Kelly Marcom,¹ Anil Potti,¹ Michael Datto,¹ John Olson,¹ William Barry,² Joseph Geradts,¹ Holly Dressman,³ Jeffrey Marks,¹ Traci Foster,¹ Lawrence Marks,¹ Sujata Ghate,¹ Robert Annechiarico,² Kirk Gray,² Geoff Ginsburg,³ and Joseph Nevins³

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Background and Objectives: Gene expression profiling has shown promise for dissecting breast cancer heterogeneity. This technique can more accurately assess prognosis and make predictions regarding response to therapy. Our approach is to make use of genome-scale gene expression data derived from cancer cell lines and tumor biopsy samples to develop expression signatures that can predict likely response to individual therapies. The preoperative setting provides an opportunity to validate genomic signatures predicting response to specific chemotherapies. Our goal was to: (1) develop the infrastructure to execute a trial using fresh-frozen tissue for genome-scale expression analysis; and (2) design a prospective preoperative trial using genome-scale expression data to predict response to doxorubicin or docetaxel-based chemotherapy.

Methods: Within the Duke Institute for Genomic Sciences and Policy (IGSP), the Clinical Genomics Services Unit (CGSU) has been organized to support processing fresh tissue to obtain genome-scale expression data. A multidisciplinary team of molecular biologists, biostatisticians, bioinformaticians, pathologists, clinical research staff, and clinicians was assembled to develop a prospective randomized clinical trial design for validating the predictive signatures developed in the IGSP.

Results: The CGSU has developed a quality-assured CLIA-compliant infrastructure for obtaining, analyzing, and making treatment assignments using genomic signatures. The prospective trial has received regulatory approval and is being extended to multiple institutions. The details of this infrastructure as well as the details of the clinical trial design and implementation will be presented.

Conclusions: The infrastructure established will allow the efficient execution of a prospective clinical trial using real-time assessment of genome-scale expression profiling. While the current trial will assess the ability of signatures to predict sensitivity to anthracyclines and taxanes, it will also demonstrate the means for incorporating detailed biologic assessment of individual patients' cancers into treatment assignment. As the heterogeneity of breast cancer is increasingly appreciated, this strategy will become critical to optimizing treatment for all patients with breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0394 and The V Foundation for Cancer Research.

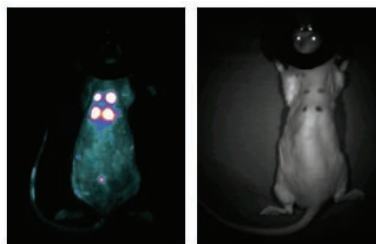
DETECTION AND DIAGNOSIS II

Poster Session P42

P42-1 NEAR-INFRARED FLUOROPHORE-LABELED TISSUE TRANSGLUTAMINASE SUBSTRATES FOR TUMOR BOUNDARY IMAGING

Chia-Pin Pan, Khalid Amin, Yihui Shi, Stephanie Olson, Jeanne P. Haushalter, Charles S. Greenberg, Zishan Haroon, and Gregory Faris
SRI International

Tumor boundary detection and monitoring are vital for diagnosing cancer progression and for effectively treating cancer. Tumorigenesis requires recruitment of new blood vessels for nutrition and extracellular matrix (ECM) for supporting the tumor tissues. This process is similar to wound healing, but instead of reaching the resolution phase at the end of healing, growing tumors continue to present the wounding stimulus and the process is never completed. In the initial phase of wound healing, blood or interstitial fluid leaks from vascular space into the extravascular compartment, leading to the formation of the provisional ECM. Transglutaminase, TG, catalyzes the formation of ϵ -(γ -glutamyl) lysine bonds between matrix proteins and stabilizes ECM. While plasma TG, Factor XIII, is activated during the fibrin clot formation, tissue TG (tTG) is expressed and involved in rat dermal wound healing and angiogenesis. tTG is also found in human and murine breast tumors and is active at the border between normal and malignant tissues, helping to form neovasculature and stabilize ECM. Thus, mapping TG activity could provide a unique mechanism to image the tumor boundary. Here, we prepared two near-infrared fluorescent-labeled TG substrates, fibrinogen and heptapeptide NQEQVSP. Both wound healing and tumor models are being tested. The preliminary results are presented. Amine-reactive HiLyte Fluor™ 750 was conjugated to fibrinogen and NQEQVSP, respectively. The home-built imaging system consists of two high-power 735-nm LEDs and an Apogee Alta-U6 monochrome CCD digital camera. Chroma HQ710/75x filters were placed in front of the LEDs to cut off the excitation above 750 nm. A Chroma HQ780LP and a Schott RG665 filter were used to eliminate scattered excitation light. The system was controlled by a LabVIEW program. Preliminary animal images of wound healing with female Fisher rats are shown in the figure. After shaving, four wounds were created by sterile 5 mm biopsy puncher on the upper dorsal region. A single dose of 250 μ L HiLyte Fluor™ 750-labeled fibrinogen ([fibrinogen] = 4 μ M, Dye/protein = 3) was injected via tail vein 24 hours after wounding. The figure clearly shows that labeled tTG substrate was presented in the wound regions 24 hours after injection and started to degrade along with experimental progression. In conclusion, we prepared two functional NIR fluorescence tTG substrates. The preliminary results showed that the labeling strategy is promising.



Fluorescence and white light images of wound healing model in rat

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0386; National Cancer Institute; and National Science Foundation.

P42-2: POLYMER ASSEMBLY IN TUMORS TO DETECT BREAST CANCER

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The anticancer drugs and fluorescent contrast agents used for cancer treatment and imaging exhibit extremely poor tumor-targeting properties. The purpose of the present work is to develop a method to detect breast cancer with greatest possible accuracy, smallest possible tumor size, and least stress and inconvenience to the patient. In the present work, it is proposed to combine different techniques into a three-step procedure to accomplish this goal. In the first step, a polymer conjugate [polymer-PNA conjugate (peptide nucleic acid)] is administered, which is retained in the tumors due to the enhanced permeability and retention (EPR) effect. The complementary PNA sequence (PNA-dye) is administered in the next step, which is cleared from the body and blood due to its smaller size but retained in the tumors owing to the base pairing between complementary PNA sequences. This places the marker molecule in the tumors, and the process can also be utilized for signal amplification. Finally, the signal is detected noninvasively.

We initially focused our attention toward establishing the mouse model of breast cancer. Instead of using immunocompromised mice that are expensive and difficult to care for, a 4T1 animal model was developed. The 4T1 model was chosen because 4T1 cells can be easily transplanted into the mammary gland, and the progressive spread of 4T1 metastases to the draining lymph nodes and other organs is very similar to that of human mammary cancer. In our model, 4T1 cells in logarithmic growth phase were harvested and injected subcutaneously into the mammary gland of 8-week-old BALB/c female mice (Jackson Laboratories). Cells injected per mouse were 1.25×10^5 , $2.05 \times$

10^5 , 5×10^5 , and 10^6 , respectively. Animals were monitored daily for tumor onset, and the tumor size was recorded daily after reaching measurable dimensions. Tumor size was calculated either by $0.5 \times \text{length} \times \text{width}^2$ or weighed after excision at the end point. Body weight was also monitored weekly. The experiment was terminated at 16 days after cell inoculation. The Leu-Gly-Doxorubicin conjugates were prepared and subsequently pegylated using poly(ethylene glycol) polymer of three different sizes (5, 10, and 30 kDa). Hilyte-labeled polymers were also prepared using polymers of three different sizes as described earlier. These conjugates were injected i.v., and their accumulation in tumor tissues was monitored noninvasively by using the skinskan instrument.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0657.

P42-3: HER2/neu-TARGETED GOLD NANOPARTICLES CONTRAST AGENTS FOR MAMMOGRAPHY AND TOMOSYNTHESIS

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Molecular imaging agents targeted to bind to specific ligands have been proposed for many imaging modalities. We are developing bioconjugated gold (Au) nanoparticles (NP) imaging agents for use in conjunction with digital mammography and breast tomosynthesis. We are currently studying the feasibility of mammographic molecular imaging using an anti-HER2 antibody (an antibody that binds to HER2/neu) conjugated to Au-NP.

Au-NP are exceptionally attenuating at mammographic energies; thus, even very low concentrations should result in significantly improved lesion conspicuity. To achieve a 5% change in attenuation at 17.5 keV (Mo K α) would require ten 100 nm diameter Au-NP per cell; this would allow the detection of a 5 mm diameter tumor at a clinical mammographic dose (1.8 mGy).

To date, 13 nm and 100 nm Au-NP have been successfully fabricated using the Turkevich method. Briefly, an aqueous chloroauric chloride (HAuCl $_4$) solution is brought to a rolling boil with vigorous stirring in a 1 L round-bottom flask equipped with a heating mantle and a condenser. The solution is rapidly reduced by an aqueous solution of sodium citrate. The Au-NP size is determined by the HAuCl $_4$ /citrate ratio. The size of the resulting Au-NP has been measured by dynamic light-scattering, UV-Vis spectroscopy and small-angle x-ray scattering. Labeling of the Au-NP is ongoing. Two cell lines have been obtained; the BT-474 cell line overexpresses the HER2/neu antigen while MDA-MB-468 has a normal antigen expression. We propose to study the feasibility of mammographic molecular imaging through in vitro "mock tumor" studies.

The successful translation of molecular imaging to mammography and digital breast tomosynthesis would allow clinical molecular imaging of the breast. The combination of such contrast agents with dual-energy subtraction breast tomosynthesis should allow high-resolution cross-sectional molecular imaging in vivo and trivial fusion of functional and anatomic images.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0618.

P42-4: IMAGING OF EP-CAM POSITIVE METASTATIC CANCER IN THE LYMPH SYSTEM

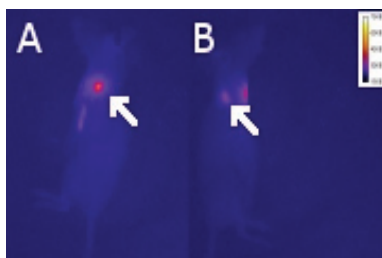
Kristen Emily Adams and Eva M. Sevick
Baylor College of Medicine

The majority of cancer mortalities occur not from the primary tumor but rather from distant metastases. Since the lymph system provides a route for the spread of metastatic cancer cells, it is not surprising that lymph node status serves as the primary prognostic indicator in most cancers. Currently, occult lymph node staging requires surgical removal of lymph nodes for subsequent biopsy, which in itself has significant morbidity. More recently, the importance of assessing "nanometastases" for accurate staging of breast cancer has been demonstrated. Developing a noninvasive imaging method for accurate and sensitive nodal staging should eliminate the deleterious effects of axillary node resection currently used for staging in breast cancer. Results from this study should eventually be used to simplify nodal staging of all epithelial cancers.

A unique imaging agent was developed to sensitively identify metastatic tumor cells within the lymph nodes of cancer patients, including breast cancer patients. This agent is an established humanized antibody against the epithelial cell adhesion molecule (Ep-CAM) that is dual-labeled with a near-infrared fluorescent dye and a radiotracer for optical and nuclear imaging of epithelial cell-based cancers. This agent has been used to identify epithelial cancer cells in vitro and in vivo in the lymph nodes of mice. Breast cancer cells were inoculated into the left mammary fat pads of mice and allowed to develop tumors for 2 weeks. Then the imaging agent was delivered intradermally and mice imaged over time to determine if the tumors spread to the left axillary node with the right axillary lymph node as a contralateral control. A pseudocolor image of the 24-hour imaging time point is shown for one mouse in Figure 1. The left axillary

lymph node appears swollen and more fluorescent than the right, which could indicate the presence of tumor cells within the left lymph node. Histological and immunochemical studies are under way currently to confirm the presence of tumor cells within the lymph nodes.

This work aims to improve the methodology for and accuracy of diagnosing cancer-positive lymph nodes. Better diagnoses could increase survival by detecting more true positive nodes, which can then be removed while reducing adverse affects by correctly identifying the negative nodes that can be retained and should contribute to improved overall lymph function.



Pseudocolor image of left (A) and right (B) sides of a mouse with the axillary node locations indicated with arrows. The left lymph node appears swollen and more fluorescent than the right, possibly indicating the presence of tumor cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0547.

P42-5: PET-MRI DUAL MODALITY TUMOR IMAGING USING CONJUGATED RADIOLABELED IRON OXIDE NANOPARTICLES

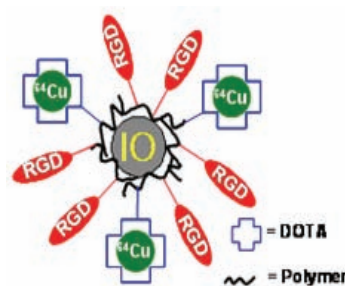
Xiaoyuan Chen and Ha-Young Lee
Stanford University School of Medicine

While simultaneous PET-CT imaging is already being used on a routine basis in clinical oncology, the combination of PET with MRI may also offer several advantages. The greatest advantage of performing combined PET-MRI is that it should theoretically be possible to obtain "perfect" spatial registration of molecular/functional PET and anatomical/functional MRI. In addition to accurate functional and anatomic localization, highly accurate image registration offers the possibility of using MR images to correct for PET partial volume effects and aid in PET image reconstruction. There have already been prototype PET-MR systems successfully implemented for small animal imaging. The purpose of this study was to develop a bifunctional iron oxide (IO) nanoparticle probe for positron emission tomography (PET) and magnetic resonance (MR) imaging of tumor integrin $\alpha_v\beta_3$ expression.

Method: Poly(aspartic acid) (PASP)-coated IO nanoparticles (PASP-IO) were synthesized using a co-precipitation method, and particle size and magnetic properties were measured. A phantom study was used to assess the efficacy of PASP-IO as a T_2 -weighted MR contrast agent. PASP-IO nanoparticles with surface amino groups were coupled to cyclic RGD peptides for integrin $\alpha_v\beta_3$ targeting and macrocyclic DOTA chelators for PET imaging after labeling with ^{64}Cu . IO nanoparticle conjugates were further tested in vitro and in vivo to determine receptor targeting efficacy and feasibility for dual PET/MR imaging. Results: PASP-IO nanoparticles made by single-step reaction had a core size of 5–7 nm with a hydrodynamic diameter of ~40 nm. The saturation magnetization of PASP-IO nanoparticles was ~117 emu/g Fe and the measured r_2 and r_2^* were 105.5 and 165.5 (s mM) $^{-1}$, respectively. DOTA-IO-RGD conjugates bound specifically to integrin $\alpha_v\beta_3$ in vitro using a cell binding assay and Prussian blue staining. Both microPET and T_2 -weighted MR imaging showed integrin-specific delivery of conjugated RGD-PASP-IO nanoparticles and prominent reticuloendothelial system (RES) uptake.

Conclusion: We have successfully developed an iron oxide-based nanoprobe for simultaneous dual PET and MR imaging of tumor integrin expression. The success of this bifunctional imaging approach may allow for earlier tumor detection with a high degree of accuracy and provide further insight into molecular mechanisms of cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0665.



Dual PET/MRI probe based on polyaspartic acid (PASP)-coated iron oxide (IO) nanoparticles.

P42-6: OPTIMIZING AN ELISA MICROARRAY PLATFORM FOR THE UTILIZATION AND DEVELOPMENT OF MULTIPLEX BREAST CANCER SCREENING

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Studies correlating gene expression patterns between breast cancer subtypes at the tissue level suggest that a unique signature of secreted proteins in serum could be used for early detection. We therefore are developing an ELISA microarray platform with the long-term goal of being able to undertake multiplex analysis in large sets (i.e., thousands) of clinical samples to provide an initial validation of the utility of a panel of markers. As part of this effort, we have developed a sophisticated bioinformatics tool for the rapid processing of ELISA microarray data (ProMAT) and a MIAME-compliant database for the management of experimental data and metadata (ELISA-BASE). We are also integrating an internal calibrant in this platform for data normalization with the goal of reducing variability across chips. Our prototype chip with 24 assays was used to optimize assay conditions for the high-throughput multiplex format. We evaluated this chip for assay cross-reactivity or interference. We found that assay cross-reactivity was rare when very low concentrations of reagents were used. We did find that reagent contamination with undesired antigens could be a problem. We have used this chip to screen human serum samples and conditioned medium from cultured human mammary epithelial cells. The studies with serum demonstrate that variability in biomarker concentrations within an individual over time are much less than inter-individual variability. Overall, this ELISA microarray platform appears to be a promising tool for evaluating biomarker profiles.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0132 and National Institutes of Health (CA117378 and EB006177).

P42-7: INTRADUCTAL APPROACH TO BREAST CANCER: UTILIZING DUCTAL LAVAGE TO INVESTIGATE THE RESTING BREAST

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¹Dr. Susan Love Research Foundation and ²University of California, Los Angeles

Background: After initial funding by the DOD in 1996 to develop the intraductal catheter, the Dr. Susan Love Research Foundation has continued to explore the intraductal approach to breast cancer risk detection and prevention. To elucidate the anatomy and physiology of the ductal systems, we initiated a study focused on cytology and hormone levels within different ducts in a breast at a 6-month interval to determine the reproducibility of the findings and the tolerability of the procedure.

Methodology: One hundred women non-selected for risk, volunteered to undergo ductal lavage in this IRB-approved study. Three ducts were lavaged in one breast and matched ducts repeated after 6 months. Nipple aspiration was attempted initially; however, if none was obtained the 2 investigators/surgeons still proceeded to cannulate 3 fluid or non-fluid yielding ducts. An intranipple lidocaine injection was used to anesthetize the area and relax the sphincter of the lactiferous sinus. This also distended and flattened the nipple making the orifices more apparent. After initial data suggested that the cytology and hormone levels of each duct were independent, ductoscopy was added to the procedure in an attempt to identify perforations and contamination of lavage with stromal fluid. Real-time ultrasound was subsequently performed during lavage for further anatomical confirmation. All three procedures on three ducts were easily accomplished within an hour.

Results: A total of 106 women have completed their first visit, 66 to date have come for their repeat visit. Five women declined the second visit due to discomfort; 2 were lost to follow up. Our mean 24-hour pain score was 1.5 (range 1-10). Only three had an initial score greater than 5. To date we have lavaged a total of 318 ducts in 106 women of whom 36% were NAF yielders. A total of 99% of ducts were recannulated. Our preliminary data shows that the 3 ducts from one woman are cytologically and hormonally independent – breast interclass correlation coefficients (ICCs) were zero. The yield of epithelial cells significantly decreased ($p=0.01$) on the second lavage while histiocytes did not. Cytological atypia was not constant over time and diminished over 6 months. Premenopausal women (53%) had significantly more histiocytes than postmenopausal women ($p=0.009$) and premenopausal women with atypia were significantly more likely to have histiocytes than those without atypia. Ducts that were noted to be perforated were significantly ($p=0.02$) more likely to have an insufficient number of cells for diagnosis.

Conclusions: Women are willing to participate in this type of basic research on the resting breast and to return for follow-up. Using real-time sonoductography we have been able to document duct or sinus distention, perforation, or stromal pooling. Each duct was found to be independent with no correlation seen in the variables studied.

Cytology is an unreliable marker in breast ductal fluid and is not reproducible over time. Further research to identify other cell and fluid markers and to understand the pathophysiology of cellular atypia, ductal epithelial regeneration, and breast cancer risk is planned.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4281; Avon Foundation; S. Mark Taper Foundation; and American Breast Cancer Foundation.

P42-8: DEVELOPMENT AND CHARACTERIZATION OF A DEDICATED COMPUTED MAMMOTOMOGRAPHY SYSTEM

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Screening of breast cancer with X-ray Mammography (XRM) has been extensively utilized in the USA, particularly because it is the second leading cause of cancer death in women. However, XRM has certain disadvantages including a high false negative rate, breast compression and related patient discomfort, higher breast dose, and low positive predictive value, especially for radiographically dense breasts. In this study, we developed a novel 3D dedicated breast Computed mammoTomography (CmT) solution for improved lesion detection at doses lower than XRM and without breast compression. The objectives of the work were to (1) *Implement the initial system*: design, construct, and automate the prototype system by implementing computer-controlled synchronization between rotation stage motor, gantry motor, anode rotor, x-ray generator, and detector controller; (2) *Evaluate unique acquisition geometries*: through a unique 3D tilting capability, develop trajectories to improve sampling closer to the chest wall; (3) *Evaluate system for variety of breast sizes, compositions, and lesion sizes*: evaluate system performance for a variety of breast sizes, shapes, and composition, and lesion sizes using signal-to-noise ratio (SNR) as well as contrast-detail observer studies. The dedicated computed mammotomography idea has been taken from concept to prototype implementation of a fully functioning and automated system. The system has been implemented with the unique capability to perform an infinite number of complex 3D orbits that has proven to significantly reduce cone-beam distortion as well as overcome patient/bed geometrical limitations by providing additional sampling close to the chest wall. A comparison of CmT with commercial standard mammography in an observer study indicated that CmT performed significantly better and at doses less than dual-view mammography. This is the only dedicated 3D breast imaging system in the world with fully 3D orbit capability and the associated ability to eliminate cone-beam distortion and improve chest wall sampling. The fact that the prototype system has performed this well is highly encouraging and the author remains very optimistic about its potential for clinical use in the near future. A company has been founded based on the technology developed in this study and has successfully received SBIR and matching state funding to commercialize this technology. It is hoped that the ultimate end result of this research will be a commercially available device that will provide a valuable tool in the fight against breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0280 and National Institutes of Health.

P42-9: PHASE APPROXIMATION FOR FLUORESCENCE-ENHANCED BREAST CANCER TOMOGRAPHY

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¹Virginia Polytechnic Institute and State University and ²University of Iowa

Optical molecular imaging has been introduced to detect breast tumors, especially at their early stage. Exogenous fluorescent molecular probes are used to report tumors by emitting photons. The planer mode of fluorescence molecular imaging lacks the ability to localize and quantify the molecular probes. In contrast, tomographic techniques are aiming for reconstructing the fluorescence-labeled tumor in three-dimension. The photon transport theory is primarily applied to develop tomographic techniques. Monte Carlo (MC) methods, discrete ordinate methods, and diffusion approximation (DA) method are among the popular computational schemes for solving the radiative transport equation (RTE). MC and discrete ordinate methods both have high computational

cost, thus are inefficient when used in inverse processes. The most widely applied DA method is only valid in weakly absorbing and highly scattering media. However, the emission spectra of fluorescence probes often violate this essential assumption of the DA, resulting in low accuracy in photon propagation modeling.

We developed a photon migration model that is computationally efficient and accurate regardless of the optical properties of different wavelength of emission light in breast tissue. Instead of the almost exclusively used Henyey-Greenstein phase function, we approximate the phase function by a generalized Delta-Eddington function. The phase function is a linear combination between isotropic scattering and the strongly peaked forward scattering. The anisotropy weight, along with the photon absorbing and scattering, characterize the optical properties of breast tissue. These optical coefficients can be determined in vivo using optical tomography techniques. With the approximated phase function, the RTE is transformed to the phase approximation (PA) formulation.

We conduct extensive numerical experiments to validate the phase approximation model. The example simulation results are based on a cylindrical phantom of 20mm in height and 20mm in diameter, which contains a spherical lesion with radius of 1 mm. We compare the accuracy of PA model to that of DA and MC while MC serves as the golden standard of photon fluence rate at the boundary. The numerical simulations suggest that the phase approximation allows accurate modeling for a wide range of optical properties. Such merit not only enhances the current fluorescent-enhanced optical tomography, but also enables many multiprobe and multispectral applications for clinical and preclinical optical imaging for breast cancer diagnostics and research.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0461.

P42-10: THREE-DIMENSIONAL COMPUTER-GENERATED BREAST PHANTOM BASED ON EMPIRICAL DATA

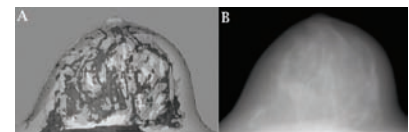
Christina Li,¹ W. Paul Segars,¹ Alexander I. Veress,² John M. Boone,³ and James T. Dobbins¹

¹Duke University Medical Center, ²University of Utah, and ³University of California, Davis Medical Center

Background and Objectives: Computer phantoms are becoming an essential tool for use in medical imaging research. Computer phantoms are advantageous in that they provide a known truth from which to evaluate imaging devices and techniques. The four dimensional (4D) non-uniform rational b-splines (NURBS) based Cardiac-Torso (NCAT) phantom was developed by Segars et al., to provide a realistic and flexible anatomical and physiological model of the human torso for use in nuclear medicine research. However, the female anatomy of the NCAT phantom only uses a simple outer surface to model the breast and does not include any detailed structures. As a result, the NCAT phantom is limited in its application to breast imaging research. The goal of this work will be to create a detailed three dimensional (3D) computer generated breast phantom based on empirical data using a combination of NURBS and subdivision surfaces (SD). The phantom will be incorporated into the 4D NCAT phantom to make it applicable to breast-imaging research.

Method: Twenty high-resolution breast CT datasets were acquired with a prototype-dedicated breast CT system. Before segmentation, the datasets were processed with a noise-reduction routine developed in our lab. Segmentation of the breast surface, pectoral muscle, and fibroglandular tissue was done using a combination of filtering, k-means clustering, morphological operations, and pseudo region-growing. NURBS surfaces were used to model the breast surface and pectoral muscle, which are concave anatomical structures. Due to its arbitrary topological structure, the fibroglandular tissue was modeled using subdivision surfaces.

Results: Figure A shows a 3D rendering of an initial phantom created based on one set of breast CT data. To illustrate the potential of the new breast phantom to produce realistic imaging data, x-ray projection images were generated using a unique analytic projection algorithm that accurately models the x-ray CT imaging process (Figure B).



(A) 3D rendering of breast tissue and
(B) projection of simulated breast

Conclusions: The final models will have a more precise representation of the internal breast structures and will be scaleable in terms of size and density. To illustrate the utility of the phantoms, we will simulate imaging data from various modalities currently used in breast imaging. The incorporation of a detailed breast model into 4D NCAT will provide an important tool in breast imaging research. It has enormous potential to investigate current and emerging techniques used in the diagnosis of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0732 and National Institutes of Health (R01 CA80490).

P42-11: MULTI-PROJECTION CORRELATION IMAGING AS A NEW DIAGNOSTIC TOOL FOR IMPROVED BREAST CANCER DETECTION

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Background and Objectives: In this study, we are investigating the feasibility of multi-projection breast Correlation Imaging (CI) as a technique to improve early detection of breast cancer. In CI, a plurality of digital radiographic projections of the same patient are acquired within a short interval of time from slightly different angles. Information from each projection image is combined to determine the final diagnosis. As a key step toward determining the clinical feasibility of CI, the acquisition scheme of CI was first optimized to yield its maximum possible diagnostic performance. Next, the performance of the optimized system was compared with that of standard planar mammography to demonstrate improvement in breast cancer detection via CI.

Methodology: Eight mastectomy specimens were acquired and imaged using an IRB-approved protocol. Under this protocol, 25 angular projections of each of the specimens were acquired at 6.25 times the standard clinical dose level. Twenty-five lower dose levels were then simulated using a noise simulation algorithm. 84 ROIs of size 100×100 were then extracted from each of the 25 angular projections, resulting in a database of $84 \times 8 = 672$ ROIs for each angular projection. These ROIs were then supplemented with a 3 mm mass. Next, an approach based on Laguerre-Gauss channelized Hotelling observer was developed to assess the detectability of the mass in terms of Receiver Operating Characteristic (ROC) curves. Two methodologies were developed to integrate results from individual projections into one combined ROC curve as the overall figure of merit.

To optimize the acquisition scheme, all of the three components of acquisition, namely, total acquisition dose, number of projections, and their total angular span were systematically changed to investigate which one of the many possible configurations maximized the area under the combined ROC curve (AUC). Optimization was investigated under two acquisition dose conditions corresponding to a fixed total dose delivered to the patient and a variable dose condition, based on the number of projections used.

Results: Per preliminary results, the detectability of the simulated mass was found to be dependent on the number of projections used, the total angular span of those projections, and the acquisition dose level. In the case of dose dependency on the number of angular projections, the detectability increased with the number of projections before approaching an asymptote at 11 to 17 projections for an angular span of about 45° . When the total dose was held constant, the detectability approximately followed a bell curve as a function of the number of projections with the maximum between 8 and 16 projections spanning angular arcs of about 23° to 45° , respectively.

Conclusions: In this study, we developed a framework to optimize the geometry of acquisitions of a multi-projection Correlation Imaging (CI) system by combining information from its multiple projections. It was found that the detectability of an embedded mass increased by fusing information from multiple projections indicating potentially improved breast cancer detection using a multi-projection CI system.

Most importantly, the framework developed in this study highlights the inherent information content of a multi-projection scheme and thus, by including reconstruction, may be extended to optimize other multi-projection imaging systems such as digital breast tomosynthesis.

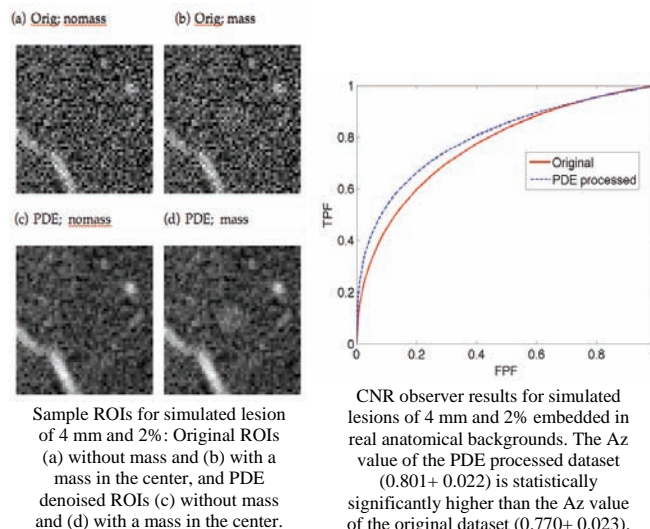
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0449.

P42-12: IMPROVED MASS DETECTABILITY IN DEDICATED BREAST CT BY APPLYING NOVEL VOLUME NOISE REMOVAL TECHNIQUES

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Dedicated breast CT imaging is a novel breast imaging modality, which may improve lesion detection while using the same radiation dose as compared against conventional mammography. However, since the breast CT images divide the dose of mammography among hundreds of projection views, resulting in considerable quantum noise, it is therefore desirable to reduce noise in the reconstructed breast volume without loss of spatial resolution. Several partial diffusion equation (PDE) based denoising techniques were developed for dedicated breast CT. The techniques were thoroughly evaluated based on simulation. It was found that applying a denoising technique before reconstruction provided better images than after reconstruction. In addition, a spatially adaptive PDE technique denoted by PDEtomo (which takes into the account the non-uniform distribution of the noise in the projection images after the preprocessing step) outperformed other techniques. The PDEtomo tool was analyzed for the clinically relevant task of lesion detectability in human subjects, using numerical observers and ROC analysis methodology. The preliminary ROC study showed that with a fixed size lesion in real anatomical backgrounds, PDE-denoised images had higher detectability (statisti-

cally significant), higher CNR and better qualitative appearance. The promising new techniques for volume noise removal pave the way for future implementations of dedicated breast CT.



This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0278.

P42-13: DEVELOPMENT OF MODALITY-INDEPENDENT ELASTOGRAPHY AS A METHOD OF BREAST CANCER DETECTION

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Vanderbilt University

Current guidelines for the treatment of breast cancer have led to improved disease-free survival and quality of life, but early detection remains of paramount importance in the effort to decrease the overall mortality of patients with this disease. Given the current status of available detection and screening techniques, there is merit to the active investigation of alternative methods for breast tissue examination beyond the established practices of x-ray mammography and the physical examination. Because clinical evidence suggests that elastic properties of normal breast and solid tumors can differ by at least an order of magnitude, research to couple mechanical excitation with imaging has created a family of methods known as elastography. In contrast to the predominant techniques of the field that encode the spatial displacement of a tissue using ultrasound and magnetic resonance (MR) imaging, we have investigated a novel recasting of the problem as an inverse problem methodology of iterative nonrigid, model-constrained image registration termed "modality-independent elastography" (MIE). By combining a biomechanical model with intensity-based similarity metrics and numerical optimization, MIE produces a spatial mapping of elastic modulus values, which can then be used to characterize and/or localize a lesion. Our first step in developing the MIE framework was a proof-of-concept characterization performed by testing nearly planar geometries. The two-dimensional (2D) approximation was able to discriminate single and multiple embedded inclusions within computer-generated simulations as well as in digital photographs of a deformable urethane rubber membrane. Additionally, procedures to evaluate reconstructions were formalized using an a posteriori statistical measure of localization, and our results showed that boundary condition specification to the model is a critical factor in elasticity image fidelity. Initial successes in 2D led us to address technical issues involved with the expansion and enhancement of the method to become a fully three-dimensional algorithm, particularly in parallel computing performance and pre-processing task efficiency. Simulation experiments were carried out in silico using image volumes of human breast from both MR and x-ray computed tomography scans to detect a synthetically embedded and radiographically occult lesion based on material inhomogeneity identified by MIE. Further experiments were performed on breast-mimicking phantoms fabricated from polyvinyl alcohol cryogels and ex vivo tissue samples in order to characterize the relative elasticity of two differing materials, and the reconstructed contrast values compared favorably with other accepted independent measures of mechanical properties. Finally, a prototype MIE data acquisition system has been constructed and is undergoing further refinement for preliminary clinical trials. Overall, the progress made in the course of this research has elucidated key elements for future study, and the results to date have been encouraging for the potential use of MIE as an adjunct technique for the screening and detection of breast cancer.

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P42-14: COMPARISON OF MOLECULAR BREAST IMAGING AND BREAST MRI FOR DIAGNOSTIC AND SCREENING APPLICATIONS

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Background: Mammography has long been the most reliable method for the detection of breast cancer and is the only modality proven to reduce breast cancer mortality. However, several studies have shown mammography to have reduced sensitivity in women who have an increased risk of developing breast cancer and in women with dense breast tissue, two populations in which there is much overlap. To improve early detection of breast cancer in these women, other modalities are continually being studied. Magnetic resonance imaging (MRI) has consistently been shown to have excellent sensitivity; however, its high cost and the high level of expertise required for accurate interpretation prohibits its widespread use for routine breast evaluation.

At Mayo Clinic Rochester, we have developed the technique of molecular breast imaging (MBI). This imaging method uses specialized gamma cameras to detect the preferential uptake of a radiotracer (Tc-99 m sestamibi) in breast disease. Ongoing studies have shown MBI to have both high sensitivity and specificity for small breast tumors. Unlike mammography, MBI is not affected by breast density and, therefore, may be a valuable adjunct technique for women with dense breasts. The MBI procedure is also significantly less expensive (~4-6 times less) than a bilateral contrast-enhanced breast MRI exam. The objective of this study was to compare the sensitivity and specificity of MBI to MRI in the evaluation of breast concerns.

Methods: Studies from patients who had undergone both MBI and breast MRI within a 30-day period were retrospectively analyzed. The gold standard for comparing sensitivity and specificity of MRI and MBI was either tissue pathology (needle/surgical biopsy, breast surgery) or the breast status determined at 15 months of follow-up for those patients who did not undergo biopsy/surgery.

Results: A total of 48 patients were included in the analysis. Of these, 42 patients had undergone MRI to further evaluate an area of clinical concern or to determine extent of disease, and 6 patients presented for high-risk screening MRI.

The MRI and MBI interpretations were concordant for presence of disease in 47 of the 48 patients. In the one discordant patient, 2 cancers were undetected by MBI that were detected by MRI.

A total of 54 cancers in 32 patients were diagnosed. MRI detected 53 cancers in 31 patients for a sensitivity of 98%, and MBI detected 51 cancers in 30 patients for a sensitivity of 94%. One cancer (DCIS present in all 4 quadrants) that was not diagnosed with either MRI or MBI was also occult on mammography and ultrasound.

Sixteen patients had totally negative findings, of which 9 were true negative on MRI for a specificity of 56%, and 8 were true negative on MBI for a specificity of 50%. Because the majority of patients in this study were suspected to have breast cancer, this specificity may not necessarily reflect that of a true screening population.

Further diagnostic evaluation was performed in 12 patients due to false-positive MRI findings, prompting 9 biopsies of benign areas. MBI studies were false positive in 11 patients, prompting 7 biopsies of benign areas.

Conclusion: Results from this retrospective analysis showed that MBI has comparable sensitivity and specificity to MRI. MBI may be a more cost-effective alternative to MRI, particularly in women who have increased risk and dense breasts.

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P42-15: IN VIVO OPTICAL IMAGING FOR CANCER DETECTION USING INSPIRATORY CONTRAST

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SRI International

Optical imaging of tissue can be a useful complementary technique to conventional imaging modalities used for breast cancer detection. Oxyhemoglobin (HbO₂) and deoxyhemoglobin (Hb) provide good contrast for near-infrared imaging. Tumors are characterized by hypoxia and angiogenesis where blood vessels are often branched and have chaotic growth patterns. We proposed that the endogenous contrast afforded by spectroscopic imaging of HbO₂ and Hb in combination with differential optical imaging during inhalation of exogenous vasoactive agents (hyperoxic and hypercapnic gases) could strongly accentuate the abnormal vasculature or tumors. We have established the viability of this hypothesis by performing whole-animal imaging. In this regard, human xenograft rodent (athymic nude mice) models of cancer were used in a transillumination optical system. Differential imaging was performed while the animals

inspired varying amounts of hyperoxic or hypercapnic gases. Thus, only relative changes in Hb and HbO₂ concentrations were calculated, and the difference images obtained are displayed as relative concentration maps. Results obtained from the U87 brain tumor cell line are shown in Figure 1. The dynamic response in Hb and HbO₂ concentrations in response to gas interventions for transmitted light from the tumor is dramatic. In Figure 2, we show the HbO₂ concentration maps for the same animal. An increased signal from the tumor region is clearly seen with hyperoxic gas inhalation. Future work includes expanding the imaging technique to track tumor growth for a

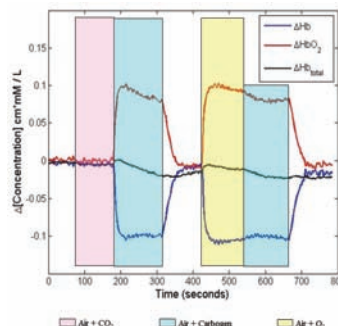


Figure 1. Changes in relative concentrations of Hb, HbO₂, and Hbtotal for a U87 tumor, subcutaneously implanted in an athymic mouse model.

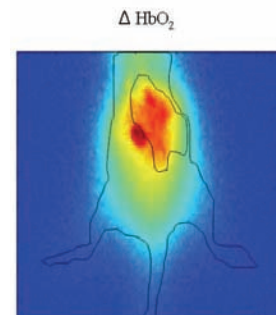


Figure 2. Near-infrared differential image of U87 tumor in mouse during hyperoxic gas inhalation. Tumor and animal outlines are shown in black.

syngeneic rat tumor (R3230 AC) and implementation of this technique to study breast tumors in preliminary clinical studies.

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P42-16: OPTICAL TOMOGRAPHY USING INDEPENDENT COMPONENT ANALYSIS

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Background and Objectives: Biomedical diffuse optical tomography (DOT) of human breast has received significant attention and development in the past two decades because of its potential to be a safe and noninvasive modality for early detection for breast cancer. The state-of-the-art diffuse optical tomography currently relies on an iterative image reconstruction approach to construct the optical properties of the interior of the medium. This approach is computationally demanding yet provides limited spatial resolution. Reconstruction of images with adequate spatial resolution and accurate localization and characterization of the targets remain a formidable task. We are developing an alternative approach, optical tomographic imaging using independent component analysis (OPTICA) for detection and localization and breast tumors. OPTICA has been developed to overcome the resolution problem of existing DOT methods and is aimed to probe cancers at their early-growth stages. Here we present the theoretical formalism of OPTICA and results from simulation and experiments on model media.

Brief Description of Methodologies: The principle of OPTICA is to first sort out the signal due to individual targets and then perform three dimensional (3D) localization and characterization for each target, using a massive data set generated by a multi-source illumination and multi-detector signal acquisition scheme. OPTICA solves the imaging problem in a series of stages. OPTICA first retrieves independent components corresponding to each target embedded inside a turbid medium, then obtains the 3D location and strength of the target from these independent components. It then reconstructs the cross-section image of the target on the transversal plane where the target is located, and finally the size and the optical properties of the target are estimated.

Results: OPTICA significantly improves the ability to detect to small/weak absorptive, scattering, and/or fluorescent targets. Using simulated data and measurements on model media with absorptive, scattering, and fluorescent targets, we achieved 3D localization of the targets with error less than 1 mm.

Conclusions: OPTICA is suited to detect small objects. Given its ability to identify low-contrast small objects, the approach is expected to be especially useful for detection of breast tumors at their early stages of growth.

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P42-17: NEAR-INFRARED TIME-RESOLVED AND SPECTROSCOPIC IMAGING FOR BREAST CANCER DETECTION

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Background and Objectives: Near-infrared (NIR) optical imaging is being pursued as a safe, noninvasive complement or alternative to x-ray mammography for detection of breast tumors with diagnostic ability. While a variety of optical imaging approaches are being developed, we have focused on a time-resolved imaging approach because of its intuitive simplicity and ability to provide simultaneous information that is useful for three-dimensional (3-D) image reconstructions. We combine the time-sliced direct two-dimensional (2-D) imaging with 2-D spectroscopic imaging to explore the diagnostic potential of our optical approach.

Brief Description of Methodologies: The experimental arrangement for time-resolved imaging used ~120-fs, 1-kHz, 800-nm light pulses from a Ti-sapphire laser system for sample illumination and an 80-ps gate-width ultrafast gated intensified camera system (UGICS) for recording direct 2-D transillumination images. The NIR spectroscopic imaging arrangement used the 1,210–1,350 nm output of a Cr⁴⁺:forsterite laser for sample illumination, a Fourier space gate selected out a fraction of the less-scattered photons, and an InGaAs NIR area camera for recording 2-D images. Ex vivo breast tissue specimens with different types of cancers were obtained from the National Disease Research Interchange and Memorial Sloan-Kettering Cancer Center under an Institutional Review Board approval from CCNY. The optical imaging results were compared with magnetic resonance imaging and histopathology findings.

Results: Figure 1 shows the typical time-resolved images of an ex vivo breast tissue sample comprising cancerous (C) and normal (N) tissues. The images recorded with earlier temporal slices of transmitted light highlighted the tumor while those recorded with later slices accentuated normal tissues. When light was tuned closer to the 1,203 nm absorption resonance of adipose tissues, a marked enhancement in contrast between the images of the cancerous (C) and normal (N) tissues was observed. Similar results were obtained for other samples with different types of breast tumors. These results correlate well with pathology and nuclear magnetic resonance-based analyses of the samples.

Conclusion: Time-resolved and spectroscopic imaging using near-infrared light has the potential to provide a breast cancer detection modality with diagnostic ability.

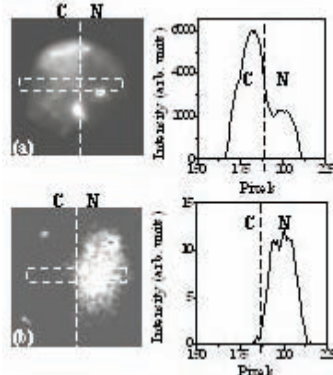


Figure 1. Time-resolved images of the sample

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0461.

P42-18: COMPUTER-ASSISTED MAMMOGRAPHY FEEDBACK PROGRAM (CAMFP): AN ELECTRONIC TOOL FOR CONTINUING MEDICAL EDUCATION

Kim Lowe and Nicole Urban
Fred Hutchinson Cancer Research Center

Background and Objectives: Our goals were to develop software to support a computer-assisted mammography feedback program (CAMFP) that could be used for continuing medical education (CME) and to evaluate if mammography interpretation performance could be enhanced by using this program. We hypothesized that providing low-volume radiologists with immediate feedback on their interpretation of difficult mammograms would improve their reading skills and result in improved sensitivity and/or specificity of mammography interpretation.

Materials and Methods: The CAMFP software was designed to run on a laptop computer (for use in this study) or over the internet. The mammograms were presented in Tagged Image File Format, which was the highest quality graphic available at the time of this study. Thirty-five low-volume mammography radiologists signed consent to participate in this institutional review board-approved study. The radiologists assessed mammograms and received feedback in 5 film interpretation sessions during an 11-month period. Films from 180 mammography subjects (89 cases and 91 controls) were used: 90 to test the hypothesis in sessions 1 and 4, and 90 for educational purposes in sessions 1, 3, and 5. During each session, the radiologist was given a simple case history and asked to record his BI-RAD assessment of each case and to indicate on the

digitized image where he believed the malignancy was located. After the assessments were made, the radiologist received immediate feedback for all cases in the set. For cases that were missed, an arrow pointed to the suspicious area or lesion and the true finding was described. To ensure adequate power to detect changes in the most clinically relevant outcomes, the study was designed using sensitivity and specificity as the basis for evaluation (Pepe 1997, *J Clin Epidemiol*). Since sensitivity and specificity tend to be inversely related, a bivariate approach was used to account for the lack of independence inherent in these measures.

Results: As described by Urban (2007, *Acad Radiol*), the interpretation of the mammograms was influenced by the CAMFP intervention and resulted in increased sensitivity (Δ sensitivity = 0.086, $p < .001$) and decreased specificity (Δ specificity = -0.057, $p = .04$) between the baseline and follow-up sessions. Variability in interpretation among radiologists also decreased after the training session ($p = .035$).

Conclusion: CAMFP provides an example of a software program that can provide a large number of training films together with immediate feedback regarding mammography interpretations. The CAMFP intervention improved sensitivity and decreased variability among radiologists' interpretations. Dissemination via the web is possible using digital mammography and the CAMFP format could contribute a valuable opportunity for radiologists to practice reading difficult films while meeting the CME requirement set forth by the U.S. Food and Drug Administration's Mammography Quality Standards Act (MQSA). CAMFP was initially designed to be used as a continuous teaching tool for radiologists in low-volume isolated practice areas. However, since it is a computer-based format that can be easily obtained over the internet, it could contribute to CME programs that are used by radiologists with varying experience levels.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6288 and National Cancer Institute.

P42-19: OPTIMIZATION OF A DUAL-ENERGY CONTRAST-ENHANCED TECHNIQUE FOR A PHOTON COUNTING DIGITAL BREAST TOMOSYNTHESIS SYSTEM

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Breast tumor growth and metastasis are accompanied by neo-angiogenesis. Elucidation of tumor vasculature kinetics is vital for identifying and differentiating breast cancers. Contrast-enhanced digital breast tomosynthesis (CE-DBT) is an x-ray imaging technique that generates a three-dimensional representation of the breast vasculature. This project focuses on the optimization of a dual-energy contrast-enhanced (DE-CE) subtraction technique for DBT. The imaging system consists of 48 linear photon-counting detectors that are precisely aligned with the focal spot of the x-ray source. The x-ray source and the digital detectors are translated across the breast in a continuous linear motion; each linear detector collects an image at a unique angle. The imaging system acquires low- and high-energy images simultaneously in a single scan by differentially filtering the x-ray beam with a pre-patient collimator. Simultaneous image acquisition minimizes the risk of patient motion but requires the low- and high-energy images to be acquired with the same target material and kV; imaging is performed with a W target at 49 kV. Sn filters (K-edge at 29.2 keV) are used to obtain low-energy images, and Cu filters are used to acquire high-energy images. System design and technical acquisition parameters are optimized using a theoretical model of the imaging system. Two system configurations are considered. In the first system, configuration, 24 Sn and 24 Cu (1:1) projection images are obtained. In the second system, configuration 16 Sn and 32 Cu (1:2) projection images are obtained. Identification of the optimal Sn and Cu beam filtration was performed. Contrast-enhanced images are obtained by applying a weighted subtraction to the logarithm of the Sn and Cu images. Optimal tissue weight factors, w_t , were determined that deliver optimal iodine-enhanced images. Optimal mean glandular dose (MGD) allocation between the Sn and Cu images was determined by calculating signal-difference-to-noise ratios (SDNR) between flat-field breast backgrounds and iodine enhancement as an objective measure of image quality. The analysis was performed as a function of breast thickness for a total MGD of 1 mSv within the heat loading and cooling capabilities of the x-ray tube. Tissue weight factors w_t depend on filter combination, breast thickness, and breast composition. The maximal SDNR is also dependent on the MGD fraction allocated to the Cu images. The optimized 1:1 system configuration uses 0.130 mm Sn and 0.135 mm Cu filters; the optimized 1:2 system configuration uses 0.090 mm Sn and 0.170 mm Cu filters. SDNR is up to 11% higher for the 1:2 system configuration. Our findings provide insight into the factors governing DE imaging performance and are important for the development of clinical DE-CE imaging systems.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0613.

P42-20: DEVELOPMENT AND OPTIMIZATION OF A DEDICATED, HYBRID DUAL-MODALITY SPECT-CMT SYSTEM FOR IMPROVED BREAST LESION DIAGNOSIS

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¹Duke University Medical Center and ²Duke University

Independently developed SPECT (single photon emission computed tomography) and CMT (computed mamtomography) sub-systems are integrated onto a single gantry to allow 3D volumetric functional/anatomical imaging of a pendant, uncompressed breast in the common field-of-view of both systems (Figure 1). Fused images can potentially provide more valuable clinical information for evaluation of cancerous diseases than the independent systems alone.

The fully 3D SPECT subsystem permits complex acquisition trajectories around the breast to avoid physical hindrances, overcome distortions due to inadequate sampling, and allow detection of lesions in the chest wall. The CMT subsystem, restricted to circular rotation, has an offset geometry to allow imaging wide range of breast sizes. The quasi-monochromatic cone-beam x-ray source allows for reduced radiation dose and increased contrast between similar soft tissue attenuation coefficients. Geometric and anthropomorphic phantoms using fiducial markers were acquired to study the sampling/resolution properties and demonstrate image fusion. In addition, a preliminary investigation on the clinical performance of the hybrid system was done by imaging a woman with confirmed breast cancer.

Images of geometric and breast phantoms were easily registered despite the slight distortion and artifacts in CMT images that possibly resulted from insufficient sampling due to the tilted and offset geometry. The initial hybrid imaging human subject study showed that SPECT images can easily see through the chest wall. Physical system constraints limited visualization of the chest wall in the CMT images, which can potentially be overcome with improved patient positioning (Figure 2).

The implementation of this first dedicated SPECT-CMT system promises greatly improved visualization in the detection/staging of cancer and the monitoring of treatment therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0791 and National Institutes of Health (R01 CA096821).

P42-21: ACCURATE 3D MODELING OF BREAST DEFORMATION FOR TEMPORAL MAMMOGRAM REGISTRATION

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University of South Florida

Accurate matching and measurement of breast lesions identified on multiple temporal mammographic views of breast are vital in detecting and treating breast cancer. However, lack of 3D structural knowledge and large compression of breast during x-ray imaging often cause mismatch among temporal mammograms, resulting in incorrect diagnosis or localization. A 3D model is strongly desired to provide accurate information about a breast's 3D geometry as well as its deformation. We have developed a 3D biomechanical model to simulate and analyze breast deformation and to register breast lesions on multiple views of mammograms.

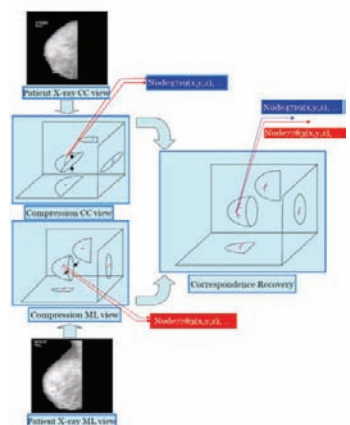


Figure 2. Recons of the (LEFT) SPECT (MIP image), and (MIDDLE, RIGHT) CT slices of the left breast

Schematic breast deformation and registration

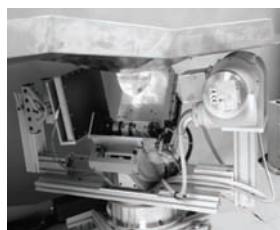


Figure 1. Prototype hybrid SPECT-CT system with customized patient bed

This study is expected to significantly improve the accuracy of matching in temporal mammograms and thus the performance of diagnosis and treatment.

Two types of 3D biomechanical models of breast deformation have been developed and numerically simulated by using rigorous finite element method (FEM). One model is developed upon magnetic resonance (MR) images of the breast, and the other one, named generic model, is based solely on mammograms. When available, MR images of breast are used to construct natural breast shape. When MR images of breast are not available, we utilize generic model of breast deformation based on breast volume estimate. Through numerical simulations of breast deformation, it is observed that a spot on mammogram corresponds to a curve in the natural breast volume. Based on this, we have developed matching methods to locate the same breast lesions in multiple views of mammograms by minimizing distances between recovered curves from the different views of mammograms.

We have experimented with the proposed methods on breast phantom images and clinical mammograms; patient images contain both cases with or without simultaneous MR images. The biomechanical model of breast deformation and registration of breast lesions on one time mammograms have been well validated. Currently, we are working on registration among multiple temporal mammograms.

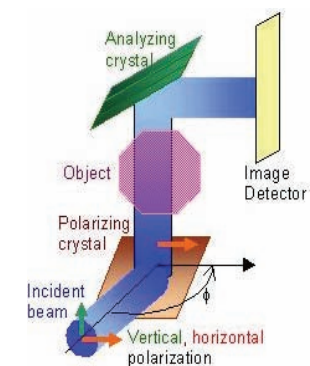
This study proposes to use a 3D finite element model with routine clinical data to assist detection and treatment of breast cancer. We have been incorporating principles of radiology, breast imaging, and computer vision to the research for accurate detection, location, and corresponding treatment of breast cancer. Moreover, the proposed modeling technique can be used in routine screening, diagnosis, and surgical planning.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0633.

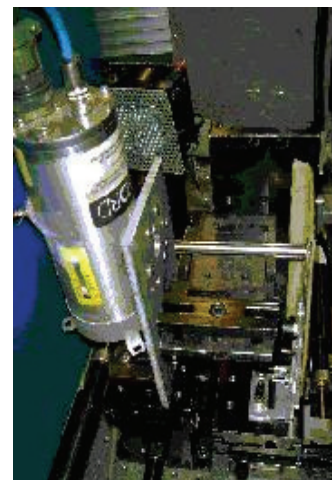
P42-22: POLARIZED X-RAY BEAM SYSTEM FOR MAMMOGRAPHY

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Contrast in x-ray images results from differences in opacity: bones are opaque and appear white; fat is less opaque and appears dark. In mammography, contrast results from very small differences between benign and cancerous tissue types, both of which are fairly transparent to x-rays. For visible light, contrast is greatly enhanced for transparent plastics, or for thin objects in optical microscopes, when the object is placed between a polarizing filter and a crossed analyzer. The primary barrier to the exploitation of these effects in x-ray imaging is the lack of convenient polarized x-ray beams. In this work, recent advances in polarized x-ray generation with conventional sources are being exploited to develop a prototype system that could be used for future testing in animal studies and for excised tissue. While synchrotron sources are naturally polarized, polarized beam production from conventional x-ray sources uses scattering or diffraction uses scattering or diffraction at 90°. Diffraction produces higher intensity, and since the angle must be maintained to within a few degrees, high-efficiency, large-area collimating optics also greatly improve the beam intensity. The choice of the diffraction crystal is a tradeoff between intensity and contrast, that is, sensitivity to depolarization effects. Simulation calculations will be verified and refined to optimize the system design.



Incident unpolarized beam is horizontally polarized after the first crystal. If the analyzing crystal is oriented with the output beam at $\phi=90^\circ$, as shown, there is no output unless the object depolarizes the beam.



A collimating lens is required to produce adequate intensity.

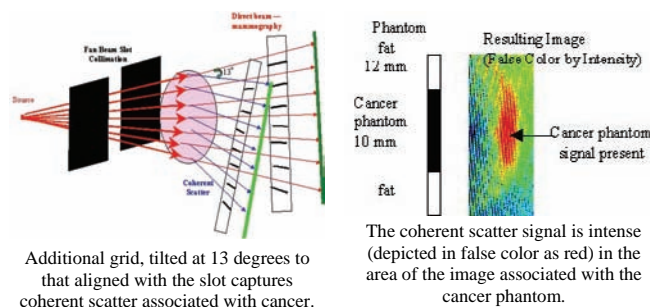
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P42-23: WIDE SLOT COHERENT SCATTER IMAGING

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Mammography is the primary screening tool for the detection of breast cancer. Contrast in conventional mammography is created when x-rays are removed from the beam by absorption and by scattering, which redirects the x-ray. The radiation scattered at large angles from the incoming beam typically has been redirected several times within the tissue and carries limited useful information. However, at low angles, coherent scatter dominates. Coherent scatter is an interference effect from nano-scale structures. Thus, it can be used to distinguish between tissue types, which have different small-scale structure. Breast carcinoma is known to have a peak coherent scattering angle at 1.6 nm^{-1} (13° for Mo K α radiation at 17.5 keV) whereas fatty tissue peaks around 9° . Conventional techniques for measuring coherent scatter use a very narrow pencil beam and area detector. The beam must be scanned in two dimensions over the entire breast with the scatter image analyzed at each point. This work demonstrated the feasibility of using a conventional slot scan mammography system to provide a spatially resolved map of a particular tissue type by placing two additional grids tilted at the desired scattering angle (e.g., 13°), as shown. Unlike improvements in the imaging system, collection of coherent scatter provides entirely new information about the local structure of breast tissue. The patient is not exposed to any additional radiation; coherent scatter is normally present but not collected. The resulting image shows that the coherent scatter from the cancer phantom was observable above the fat background. Theoretical calculations for the intensity distributions compared well to measurements. This work indicates promising potential for including coherent scatter analysis in screening mammography to improve the sensitivity and specificity.



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P42-24: INVESTIGATION OF IMAGING CONFIGURATIONS AND RECONSTRUCTION ALGORITHMS FOR DIGITAL BREAST TOMOSYNTHESIS

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Background and Objectives: Mammography has been used widely for breast cancer imaging. However, some of the 3D structural information within the breast can be obscured in mammographic projection images. Digital breast tomosynthesis (DBT) has the potential to overcome this major limitation by recovering some of the obscured information. The goal of this project is to investigate and evaluate innovative data-acquisition schemes and reconstruction algorithms to improve DBT image quality and ultimately DBT-based detection of breast cancer lesions.

Brief Description of Methodologies: In DBT, projection data are acquired only at a small number of views over a limited angular range, which are highly sparse as compared to the conventional computed tomography (CT). From sparsely sampled data, iterative algorithms can generally produce images with fewer artifacts than can analytic algorithms. In this project, we investigated and evaluated an iterative algorithm that reconstruct images through minimizing the total variation (TV) of the breast image under the DBT data constraint.

Results to Date: In the project, we investigated several issues concerning data acquisition and image reconstruction in DBT, including various imaging parameters and configurations, imaging physical factors, and reconstruction evaluation. In this abstract, we report our results on studying data-acquisition parameters such as (1) the number of views, (2) angular range, and (3) non-planar source trajectories.

In our studies, we generated data from a variety of phantoms at different numbers of views over a limited angular range for a given imaging configuration. Specifically, we generated data sets that include 11, 20, 40, and 60 views over each of the angular ranges of $\pi/4$, $\pi/2$, and π . In addition to the circular arc source trajectory, we investigated an innovative trajectory in which the source can be moved over a curved surface such as a portion of the sphere. From these data, we reconstructed images by use of the TV-

based algorithm. For comparison, we also used two standard iterative algorithms, expectation maximization (EM) and algebraic reconstruction technique (ART), to reconstruct images from these data sets.

Our results indicate that the TV-based algorithm consistently outperforms the EM and ART algorithms in terms of visual inspection and quantitative evaluation of the reconstructed images. In general, the quality of reconstructed images is improved with an increased number of views for a given angular range and a source trajectory, or an increased angular range for a given source trajectory. More interestingly, for the same number of views (i.e., the same amount of radiation dose), the non-planar source trajectory yields data containing more information than the circular arc trajectory, thus leading to images with improved quality.

Conclusion: In the work, we have investigated issues concerning data acquisition and image reconstruction in DBT. Our studies indicate that the TV-based algorithm generally yields images with higher quality than do the EM and ART algorithms. Also, data acquired with an increased number of views and/or an increased angular range can produce images with improved quality. For the same number of views (i.e., the same amount of radiation dose), data generated with the non-planar trajectory contain more information than the circular arc trajectory, thus leading to improved image quality.

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P42-25: COMPARISON AND OPTIMIZATION OF DIGITAL BREAST TOMOSYNTHESIS IMAGE RECONSTRUCTION ALGORITHMS AND IMAGING ACQUISITION PARAMETERS

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Background and Objectives: Breast cancer has been considered as a major problem and the most common cancer among women. Digital breast tomosynthesis (DBT) is a three-dimensional breast imaging method that allows the reconstruction of an arbitrary set of planes in the breast from limited-angle series of projection images. The selection of optimal acquisition parameters and reconstruction algorithm plays an important role in producing better imaging performance. However, there are several factors involved in this task and some of them are not individually independent. This project is to quantitatively evaluate the noise equivalent quanta (NEQ) characteristics of several breast tomosynthesis reconstruction algorithms and different imaging acquisition techniques for comparison and optimization purposes. It combines the modulation transfer function (MTF) of signal performance and the noise power spectrum (NPS) of noise characteristics.

Methods: A full-field Siemens prototype digital breast tomosynthesis system with pixel resolution of $85 \mu\text{m}$ was used. Seven available acquisition modes including different acquisition parameters (number of projection images, total angular range, binning/non-binning mode) and three representative fast-speed image reconstruction algorithms including point-by-point back projection (BP), matrix inversion tomosynthesis (MITS), and filtered back projection (FBP) were investigated. The MTF measurement included two parts: (1) the measured system MTF of the detector by a previously published edge method and (2) the relative reconstruction MTF associated with specific reconstruction algorithm and acquisition mode by computer ray-tracing simulation and computation. Mean-subtracted NPS results were analyzed and compared on a defined reconstruction plane for each reconstruction algorithm and acquisition mode to remove fixed pattern noise, including structured noise and system artifacts. The relative NEQ(f) along the tube's motion direction was examined to evaluate the performance of different acquisition parameters and algorithms.

Results: Compared with other imaging acquisition modes, MITS performed the best with 49 projections and $\pm 25^\circ$ total angular range. Point-by-point BP and FBP performed slightly better for non-binning modes with more projection numbers. FBP performed worse with too limited sampling in the frequency space. FBP showed better relative NEQ(f) result than that of MITS for the cases investigated. The relative NEQ(f) comparison result between MITS and FBP in this project is only limited to breast tomosynthesis imaging. It doesn't apply to other tomosynthesis applications such as chest imaging where MITS technique shows advantages in image quality and artifact reduction. Based on relative NEQ(f) analysis, FBP with larger number of projection images is the optimal breast tomosynthesis method. Considering the longer acquisition time of tomosynthesis sequence of 49 projection images, 25 number of projection images without binning is suggested as the optimal acquisition mode for FBP reconstruction.

Conclusion: Digital breast tomosynthesis is an effective technology of reconstructing three-dimensional breast images. With same exposure level to the patient, preferable reconstruction algorithm with optimized imaging acquisition techniques will provide better reconstruction images to improve early breast cancer detection.

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P42-26: COMPUTER-AIDED DETECTION OF BREAST MASSES IN DIGITAL TOMOSYNTHESIS

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The purpose of this study was to propose and implement a computer aided detection (CADE) tool for breast tomosynthesis. This task was accomplished in two stages—a highly sensitive mass detector followed by a false-positive (FP) reduction stage. Breast tomosynthesis data from 100 human subject cases were used, of which 25 subjects had one or more mass lesions and the rest were normal. For stage 1, filter parameters were optimized via a grid search. Just the CADE-identified suspicious locations were reconstructed to yield 3D CADE volumes of interest. The first stage yielded a maximum sensitivity of 93% with 7.7 FPs/breast volume. Unlike traditional CADE algorithms in which the second stage FP reduction is done via feature extraction and analysis, instead information theory principles were used with mutual information as a similarity metric. Three schemes were proposed, all using leave-one-case-out cross-validation sampling. The three schemes, A, B, and C differed in the composition of their knowledge base of regions of interest (ROIs). Scheme A's knowledge base was comprised of all the mass and FP ROIs generated by the first stage of the algorithm. Scheme B had a knowledge base that contained information from mass ROIs and randomly extracted normal ROIs. Scheme C had information from three sources of information—masses, FPs, and normal ROIs. Also, performance was assessed as a function of the composition of the knowledge base in terms of the number of FP or normal ROIs needed by the system to reach optimal performance. The knowledge base needs no more than 20 times as many FPs and 30 times as many normal ROIs as masses to attain maximal performance. The best overall system performance was 85% sensitivity with 2.4 FPs/breast volume for Scheme A, 85% sensitivity with 3.6 FPs/breast volume for Scheme B, and 85% sensitivity with 3 FPs/breast volume using Scheme C.

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P42-27: LOSS OF JNK2 SHORTENS TUMOR LATENCY AND ENHANCES TUMOR MULTIPLICITY VIA DNA DAMAGE AND ANEUPLOIDY-MEDIATED RESPONSES

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c-Jun N-terminal kinases (JNK) mediate downstream messages for a wide variety of important cancer genes including Phosphatidylinositol 3-kinase (PI3K), Rac1, Phosphatase and Tensin homolog deleted on chromosome Ten (PTEN), and Ras. These findings show that JNK conveys important phenotypic responses, and its inhibition may prove a useful therapeutic target in diseases like breast cancer. Critical to this approach, however, is an understanding of the specific contributions of the various JNK isoforms transcribed from three jnk genes. Our goal was to evaluate the function of JNK2 isoforms as targets of growth factor-mediated signaling in mammary tumorigenesis and metastasis. Here we show that systemic loss of jnk2 in the polyoma middle T antigen (PyV MT) transgenic mouse model shortens tumor latency by over 2 weeks and statistically significantly increases both tumor multiplicity and aneuploidy. Despite our findings that jnk2 loss leads to early tumor latency and aneuploidy, the PyV MT/jnk2^{-/-} tumors show lower expression of metastatic markers including osteopontin and the chemokine receptor CXCR4. We then evaluated DNA damage as a potential cause of the higher aneuploidy rate in the PyV MT/jnk2^{-/-} tumors. Compared to the jnk2 wild-type control tumors, the jnk2^{-/-} tumor samples show less H2AX phosphorylation but similar frequency of 53BP1 foci, indicating an inefficient or aberrant DNA damage response or repair mechanism. Comparative genomic hybridization (CGH) was used to compare gene alterations between the jnk2 knockout and wild-type PyV MT tumors. CGH results showed both common and unique segmental changes consistent with gene amplifications and deletions on various chromosomes.

Using mammary tumor cell lines from the same two tumor genotypes, we compared growth factor responsiveness. To this end, cells are serum starved overnight and then re-stimulated with serum and assayed in a time-dependent fashion. Notably, the PyV MT/jnk2^{-/-} cells undergo S-phase-mediated apoptosis, in contrast to the jnk2 wild-type cells that efficiently transit the cell cycle. This effect is partially rescued by re-expression of JNK2a, inhibition of ataxia-telangiectasia gene (ATM) kinase using caffeine treatment, or pharmacologic inhibition of the remaining JNK1 isoforms. These data suggest JNK2 mediates S-phase transit in mammary tumor cells. In its absence, JNK1-induced activity leads to cell death. We are currently evaluating if replicative stress plays a role in our model. Our data show an important function for JNK2 in tumorigenesis and genomic stability. While aneuploidy is thought to lead to more metastatic disease, our data thus far suggest that JNK2 mediates a variety of functions important in mammary tumorigenesis.

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P42-28: L1 ELEMENTS AND GENETIC INSTABILITY IN BREAST CANCER

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L1 elements are retroelements that make up 17% of the human genome. In addition to the 500,000 L1 elements, the machinery that drives L1 retrotransposition is responsible for insertion of an additional 14% of the genome due to Alu, SVA, and processed pseudogene insertions. Thus, L1 elements are responsible for a great deal of the genetic instability in the genome. These insertions have contributed to over 50 different human diseases. Recently we have shown that the endonuclease made by L1 elements is also responsible for a high level of DNA double-strand break activity, above and beyond their insertion ability. We have now shown that at least some of those double-strand breaks are the result of initiation of insertion by the L1 element, followed by recognition and repair of the partial insert through the nucleotide excision pathway. CHO cells defective for ercc1 support high levels of L1 retrotransposition. Co-expression of ercc1 along with the L1 retrotransposition vector results in about a 4-fold decrease in retrotransposition.

We have also shown that several breast cancer cell lines have particularly high levels of expression of full-length L1 RNAs. Thus, L1 expression in breast cancers is likely to contribute to a high level of retrotransposition events, as well as double-strand breaks in DNA that may lead to recombination events and LOH.

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P42-29: EXTRACELLULAR MATRIX SIGNALING VIA β 1 INTEGRIN REGULATES DNA DOUBLE-STRAND BREAK REPAIR

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Genomic instability and aberrant cell—extra cellular matrix (ECM) interactions are hallmarks of malignancy in glandular tissues including the breast. One cause of genomic instability is altered double-strand break repair. Double-strand breaks (DSBs) are caused by endogenous factors such as blocked replication, oestrogen intermediates, reactive oxygen species, and by exogenous factors such as ionizing radiation during cancer treatment. Breaks can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) pathways, both of which can lead to mutations depending on genomic location, presence of repeat sequences, and the sub-pathways invoked, but HR is accepted as the less error prone pathway. Apart from cell cycle effects, it is not clear what determines pathway choice in DNA double-strand break repair. Here we show that in primary mouse mammary epithelial cells, as well as in a human breast epithelial cell line, ECM signaling via β 1 integrin regulates HR of a break within a direct repeat: ECM upregulates HR if normal cell-cell junctions are present but downregulates it in single cells. This regulation is direct and independent of cell cycle effects of ECM. Furthermore, we found that formation of γ -H2AX, MRE11, and RAD51 foci in response to double-strand break formation via ionizing radiation in single nondividing human epithelial cells is attenuated by ECM signaling via β 1 integrin, whereas γ -H2AX foci formation in primary mouse mammary epithelial cells with normal junctions is upregulated, consistent with the roles of these proteins in HR. Therefore, the effects of ECM on HR are genome wide and not restricted to the repair of the single break within a direct-repeat substrate. Our results suggest that the role of ECM in tissue homeostasis includes regulation of DNA repair. The observation that ECM enhances repair in the context of tissue-like cell-cell junction formation but downregulates it in a single cell lacking these junctions suggests that ECM may function to balance processes relevant to genome stability with maintenance of tissue integrity.

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P42-30: NON-HOMOLOGOUS END JOINING IS ESSENTIAL FOR CELLULAR RESISTANCE TO THE NOVEL ANTITUMOR AGENT, β -LAPACHONE

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Commonly used antitumor agents, such as DNA Topoisomerase I/II poisons, kill cancer cells by creating non-repairable DNA double-strand breaks (DSBs). To repair DSBs, error-free homologous recombination (HR) and/or error-prone non-homologous end joining (NHEJ) are activated. These processes involve the phosphatidylinositol 3-kinase related kinase (PI3K) family of serine/threonine protein kinases: ataxia telangiectasia mutated (ATM), ATM-, and Rad3-related (ATR) for HR, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) for NHEJ. Alterations in these repair processes can cause drug/radiation resistance and increase genomic instability. β -Lapachone (aka ARQ 501), currently in Phase 2 clinical trials for the treatment of

pancreatic cancer, causes a novel caspase- and p53-independent cell death in cancer cells over-expressing NAD(P)H:quinone oxidoreductase-1 (NQO1). NQO1 catalyzes a futile oxidation of β -lapachone leading to reactive oxygen species (ROS) generation, DNA breaks, γ -H2AX foci formation, and the hyperactivation of poly(ADP-ribose) polymerase-1 (PARP-1), which is required for cell death. Here, we report that β -lapachone exposure results in the NQO1-dependent activation of the Mre11-Rad50-Nbs-1 (MRN) complex. In addition, ATM serine 1981, DNA-PKcs threonine 2609, and Chk1 serine 345 phosphorylation were noted; indicative of simultaneous HR and NHEJ activation. However, inhibition of NHEJ, but not HR, by genetic or chemical means potentiated β -lapachone lethality. These studies give insight into the mechanism by which β -lapachone radiosensitizes cancer cells and suggests NHEJ is a potent target for enhancing the therapeutic efficacy of β -lapachone alone or in combination with other agents in cancer cells that express elevated NQO1 levels.

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P42-31: Brh2-Dss1 INTERPLAY ENABLES PROPERLY CONTROLLED RECOMBINATION IN *Ustilago maydis*

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Brh2, the BRCA2 homolog in *Ustilago maydis*, functions in recombinational repair of DNA damage by regulating Rad51 and is, in turn, regulated by Dss1. Dss1 is not required for Brh2 stability in vivo, nor for Brh2 to associate with Rad51, but is required for formation of GFP-Rad51 foci following DNA damage by γ -radiation. To understand more about the interplay between Brh2 and Dss1, we isolated mutant variants of Brh2 able to bypass the requirement for Dss1. These variants were found to lack the entire C-terminal DNA/Dss1-binding domain but to maintain the N-terminal region harboring the Rad51-interacting BRC element. GFP-Rad51 foci formation was nearly normal in brh2 mutant cells expressing a representative Brh2 variant deleted of the C-terminal domain. We prepared a collection of Brh2 deletion mutants to define what features of the protein were critical in DNA repair activity. The points of chain termination in the active Brh2 variants were confined to a narrow window such that the expressed N-terminal region included the BRC element and a putative nuclear localization signal but was excluded of even a few Dss1-interacting residues. These findings suggest that the N-terminal region of Brh2 has an innate ability to organize Rad51. Survival after DNA damage was almost fully restored by a chimeric Brh2 having a DNA-binding domain from RPA70 fused to the Brh2 N-terminal domain, but Rad51-focus formation and mitotic recombination were greatly elevated. The results provide evidence for a mechanism in which Dss1 activates a Brh2-Rad51 complex and balances a finely regulated recombinational repair system.

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P42-32: FUNCTIONAL INTERACTION OF THE TUMOR SUPPRESSORS p53 AND BARD1 WITH THE POLYADENYLATION FACTOR CstF-50

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Following UV irradiation the levels of cellular mRNA are transiently decreased due to the inhibition of transcription and mRNA polyadenylation. The UV-induced inhibition of 3' processing reflects the interaction of the polyadenylation factor CstF-50 with the tumor suppressors BRCA1/BARD1 as well as the BRCA1/BARD1-mediated degradation of RNA polymerase II (RNAP II). As CstF-50 can interact with both BRCA1/BARD1 and RNAP II inhibiting or activating polyadenylation, respectively, we propose a coordinating role for this factor in the DNA damage response. Supporting this idea, cells with reduced levels of CstF show both an enhanced sensitivity to UV and reduced ability to ubiquitinate RNAP II and repair DNA.

In this study we add a new level of complexity to the cellular response to UV treatment by showing information that links RNA processing to the p53 network. Here we show that the CstF-50 factor can interact directly with the C-terminal domain of p53. We and others have shown that in response to DNA damage BARD1 not only interacts with CstF-50 inhibiting polyadenylation but also with p53 mediating induction of apoptosis. Our results indicate that the interaction of CstF-50 and p53 does not interfere with the binding between p53 and BARD1; rather these proteins form a ternary complex following DNA damage. This suggests that p53 may also participate in the coupling of 3' pre-mRNA processing to the DNA damage response. Consistent with this and correlating with the binding data, we found that the p53-CstF-50 interaction inhibits polyadenylation in vitro and that the C-terminal of p53 is necessary for the inhibition. Significantly, cells expressing different levels of p53 have different effects on polyadenylation and on UV-induced inhibition of polyadenylation. For example, siRNA-mediated knockdown of p53 in cells that express normal levels of p53, such as MCF7, resulted in extracts with levels of 3' cleavage similar to the ones observed in mock-

treated HeLa cells, which are HPV-E6 transformed and, therefore, have low levels of p53 expression. Extending these results, a previously identified p53 mutant (Ser241 Phe), which is expressed in DLD-1 cells, had a moderate effect not only on binding CstF-50 but also on the inhibition of polyadenylation. However, similar to MCF-7 cells, siRNA-mediated depletion of p53 in DLD-1 cells enhanced the levels of 3' processing, suggesting that the Ser241 might play a role in inhibiting cleavage.

Our results identify a novel 3' RNA processing inhibitory function of p53, suggesting that the p53-CstF-50 interaction contributes to UV-induced inhibition of polyadenylation and provides evidence of a link between mRNA 3' processing, DNA repair, tumor suppression, and apoptosis.

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P42-33: DSS1, A BRCA2- AND PROTEASOME-ASSOCIATED PROTEIN, REGULATES HOMOLOGOUS RECOMBINATIONAL REPAIR OF CHROMOSOME BREAKS IN HUMAN CELLS

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The breast cancer suppressor protein BRCA2 functions in homologous recombinational repair (HRR) of DNA double-strand breaks and does so, in part, through the actions of a carboxy-proximal region that binds single-stranded DNA and DSS1; the BRCA2 DNA/DSS1-binding domain (BRCA2-DBD). DSS1 (deleted in split-hand/split-foot syndrome) is a small, highly acidic protein widely conserved among eukaryotes, including some that have no BRCA2 ortholog. In *Ustilago maydis*, DSS1 interacts with the BRCA2 ortholog Brh2 and controls its function in mediating the formation of RAD51-ssDNA nucleoprotein filaments at the resected ends of double-strand breaks. We used RNA interference to deplete DSS1 in a human cell line and directly assayed the effects on double-strand break repair by homologous recombination. Analogously to dss1 mutation in *Ustilago*, reduced DSS1 expression in human cells results in severe deficiency of HRR, indicating that DSS1 contributes to the function of human BRCA2 in mediating recombination. DSS1 has also been associated with the 19S proteasome in yeast and mammalian cells. Yeast dss1 mutants are deficient in ubiquitin-mediated proteolysis and hypersensitive to DNA damage. In human cells, we find that drug inhibition of proteasomes also impairs HRR but to a much lesser extent than depletion of DSS1. Hence, a role of human DSS1 in proteolysis probably cannot account completely for the dependence of HRR upon DSS1. By immunofluorescent localization, we find that human DSS1, in addition to being a predominantly nuclear protein, is concentrated in promyelocytic leukemia nuclear bodies (PML-NB). Numerous other proteins involved in HRR have been reported to associate transiently or conditionally with PML-NB, suggesting a role for PML-NB in regulating or coordinating HRR. DSS1, however, is apparently a constitutive component of PML-NB, as it is present both before and after DNA damage, and throughout the interphase cell cycle. Whether and how the association of DSS1 with PML-NB figures in HRR are not yet clear.

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P42-34: FUNCTIONAL CHARACTERIZATION OF BREAST CANCER-ASSOCIATED MISSENSE MUTANTS OF ATAXIA-TELANGELECTASIA MUTATED

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Defects in the serine/threonine kinase ataxia-telangiectasia mutated (ATM) gene result in the rare genetic disorder ataxia-telangiectasia (A-T), which is clinically characterized as neurodegeneration, immunodeficiency, and cancer predisposition. On a molecular level, A-T cells exhibit defective DNA double-strand break (DSB) repair, checkpoint control, and apoptosis. Under normal conditions, ATM exists as an inactive dimer that dissociates into active monomers when DSBs are detected. Active monomeric ATM phosphorylates many downstream effector proteins that are involved in cell-cycle arrest, apoptosis, and DNA repair. Together these defects contribute to enhanced sensitivity to DSB-producing agents, such as ionizing radiation (IR), cellular transformation, and cancer progression.

Studies of A-T families have shown that heterozygous carriers of ATM missense mutations are predisposed to breast cancer; however, a lack of biochemical evidence prevents a mechanistic understanding of how ATM mutations contribute to cancer progression. The objectives of the proposed research are to characterize breast-cancer associated ATM missense mutants at a biochemical level. We aim to characterize mutant ATM protein both as a monomeric species as well as in a heterodimeric complex with a wild-type ATM. Analysis of monomeric proteins will help us determine whether the missense mutation disrupts substrate binding or kinase activity, while heterodimeric complex studies will aid in understanding upstream processes such as dimer formation and monomerization.

We have taken a biochemical approach to study the Ser707Pro, Val2424Gly, and Ser2592Cys breast-cancer associated ATM missense mutants, which consists of site-directed mutagenesis of the ATM gene followed by affinity chromatography purification of either monomeric or heterodimeric ATM complexes. We have devised in vitro assays to measure ATM kinase activity, substrate and MRN protein-protein interactions, and ATM dimer dissociation. Whereas other groups predominately assay ATM from immunoprecipitated extracts, we are able to reconstitute ATM activation using recombinant, purified components in vitro and are therefore able to determine precisely the defect in these mutant ATM proteins.

We have successfully constructed and purified monomeric forms of the three ATM missense mutants and have performed in vitro kinase assays. We have preliminary data suggesting that two of the mutants (S707P and S2592C) retain normal kinase activity, while V2424G is kinase-deficient. We have also purified heterodimeric mutant/wild-type complexes, which demonstrate the ability of these mutants to form complexes with wild-type ATM. Efforts to characterize these dimeric complexes are currently under way in our lab.

Based on these preliminary results, we can conclude that ATM missense mutants contribute to cancer progression through various mechanisms. For instance, S707P and S2592C may form heterodimeric complexes with wild-type ATM but may remain in an inactive-dimer after DSBs are detected, which leads to DNA damage persistence and potentially cancer. Another subset of ATM mutants, epitomized by V2424G, may simply be kinase-deficient proteins that may compete with wild-type ATM for substrates and MRN. Because this proposal focuses only on three breast-cancer ATM mutants, we believe there are mutants that do not fall into one of these two categories and likely contribute to cancer progression through another mechanism.

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P42-35: p53 AND Xpc CONTRIBUTE SIGNIFICANTLY TO CELLULAR PROTECTION FROM DNA DAMAGE

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There is a highly complex network of molecular interactions responsible for maintaining the integrity of the genome within the cell. At the center of this network is the tumor suppressor protein, p53. These data demonstrate that p53 protects normal, healthy cells from DNA damage. More importantly, the results show that p53-mediated protection from DNA damaging agents can be enhanced. We treated normal, healthy cells with selenomethionine prior to challenge with cisplatin and found that the selenium supplementation enhanced DNA repair and improved cell survival following chemotherapy. However, cells that lacked a functional copy of p53 remained sensitive to the cisplatin treatment and had no improvement in DNA repair. Furthermore, the results identify a downstream target of p53, namely Xpc, which contributes significantly to the protective response in mouse bone marrow. Xpc is a protein responsible for recognizing DNA damage, and the data reveal that mice lacking the Xpc gene had substantially greater bone marrow toxicity than normal mice.

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P42-36: FUNCTIONS OF ATRIP IN THE REGULATION OF THE DNA DAMAGE ACTIVATED KINASE ATR

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The ATR (ATM and Rad3-Related) kinase is essential to maintain genomic integrity. ATR exists in a complex with ATR-interacting protein (ATRIP). In response to DNA damage, the ATR-ATRIP complex is recruited to DNA lesions in part through an interaction between ATRIP and the single-stranded DNA binding protein RPA (Replication Protein A). We report the identification of a conserved checkpoint protein recruitment domain (CRD) in ATRIP orthologs by biochemical mapping of the RPA binding site in combination with NMR, mutagenesis, and computational modeling studies. Mutations in the CRD of the yeast ATRIP ortholog Ddc2 disrupt the Ddc2-RPA interaction, prevent proper localization of Ddc2 to DNA breaks, sensitize yeast to DNA damaging agents, and partially compromise checkpoint signaling. These data demonstrate that the CRD is critical for localization of the ATR-ATRIP complex and optimal DNA damage responses. However, the stimulation of ATR kinase activity by TopBP1 binding to the ATR-ATRIP complex does not require the ATRIP CRD. We also demonstrate that specific regions of ATRIP promote the activation of ATR by TopBP1. Thus, our results support a multi-step model for ATR activation that requires separable localization and activation functions of ATRIP.

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P42-37: HOMEOSTATIC REGULATION OF DNA DAMAGE RESPONSES BY THE WIP1 PHOSPHATASE

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After exposure to endogenous or environmental stress, the cell initiates an intricate signaling cascade by phosphorylating and activating target proteins in the DNA damage response pathway to arrest cell cycle progression, stimulate DNA repair processes, and/or initiate programmed cell death responses. While activation of the DNA damage response pathway is well studied, the mechanism by which the cell returns to a homeostatic state following completion of DNA repair remains to be elucidated.

Following DNA damage, p53 becomes activated and upregulates the *wild-type p53-induced phosphatase 1 (WIP1)* gene. WIP1, also known as the protein phosphatase 1D magnesium-dependent delta isoform (PPM1D), is a serine-threonine phosphatase of the type 2C protein phosphatase family. WIP1 is a bona fide oncogene that is amplified and overexpressed in a variety of cancers, including breast cancers, medulloblastomas, neuroblastomas, and ovarian clear cell adenocarcinomas. While it cooperates with other oncogenes to transform primary rodent fibroblasts, WIP1 depletion confers a tumor resistance phenotype in WIP1^{-/-} mice. We hypothesize that the mechanism of WIP1-mediated tumorigenesis is probably through its ability to inactivate multiple stress response pathways.

WIP1 dephosphorylates and inactivates several signaling pathways, including the ATM/ATR DNA damage response pathway (by targeting the ATM, Chk1, and Chk2 kinases) and the p53 auto-regulatory loop pathway (by targeting p53 and Mdm2). In addition, WIP1 also plays a role in inactivating DNA repair, specifically base excision repair and perhaps nucleotide excision repair. Therefore, WIP1 may function to reverse the ATM/ATR-induced DNA damage and cell cycle checkpoint responses and return the cell to a homeostatic state following completion of DNA repair.

Recent discoveries that activation of the DNA damage response is often an early marker of neoplasia and that abrogation of this response often accompanies more advanced forms of cancer indicate that WIP1 overexpression is likely to be oncogenic not only because of its p53 suppression, but also because of its general inhibition of the DNA damage response. Elucidation of additional WIP1 targets should bring further clarity to our understanding of the normal homeostatic role of WIP1 in the DNA damage response and the aberrant role of WIP1 when it is overexpressed in the cancer cell.

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P42-38: NEW HIGH-THROUGHPUT MICROSCOPY SCREEN REVEALS VARIATIONS IN THE RESPONSE OF BREAST CANCER-RELATED PROTEINS TO DIFFERENT CHEMOTHERAPEUTIC DRUGS

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Breast cancer in women between the ages of 40 – 55 is the leading cause of death in the United States each year and the second leading cause of death for all women. Greater than 250,000 women this year alone will be diagnosed with either invasive or in situ breast cancer, with approximately 16% resulting in death. Moreover, there will be an estimated 2000 cases reported in the male population this year with a mortality rate of nearly 20%. Early diagnoses and effective treatment remain the most effective ways to increase survival rates among the affected population. In addition to surgical intervention, radiation and chemotherapy regimens are included as standard treatment procedures for breast cancer. Important to our appreciation of how these treatments affect cancerous cells is an understanding of the complicated molecular events surrounding breast cancer related protein markers such as phosphorylated histone H2AX, Breast Cancer Related Protein 2 (BRCA2) and other functionally related proteins. In response to breast cancer chemotherapeutics, whose function is to destroy the genetic material (DNA) of cancer cells, these proteins form visible nuclear foci. These foci mark the location of drug-induced DNA double strand breaks, and clearance of these foci indicates the timing of DNA repair within the cellular environment. To date foci formation and clearance have been analyzed and quantified using traditional microscopy techniques requiring the production of many samples and counting of individual cells within large fields of cells. These methods can be flawed by misinterpretation of protein localization and are limited in many cases by an inability to collect statistically meaningful numbers of data samples. These problems can result in an inaccurate measure of the response of these critical proteins to various treatments. Here we employ "Blueshift technology," a high throughput microscopy screen, to undertake the first examination of large populations of human cell with respect to breast cancer associated proteins. We report the examination of multiple human cell lines, counting hundreds of thousands of cells, while simultaneously collecting data indicating the formation and clearance of

breast cancer-related protein foci as well as high-resolution microscopic images of their cellular localization. Our initial findings suggest that a subset of the chemotherapeutics tested do not affect the entire cell population and that of the affected population only about 50% actually show clearance of the foci formed in response to the treatment. While the appearance of protein foci occurs randomly throughout the cell nucleus and is dose-dependent, we find that focal clearance appears to be rapid, coordinated, and dose-independent. Additionally, the level of response for individual breast cancer related proteins as measured by foci formation and clearance varies greatly for individual drugs, with some proteins persisting in the focus state long beyond the estimated time of DNA repair. The data collected thus far suggest that the treatment of breast cancer by various chemotherapeutics as well as by ionizing radiation may in fact trigger variability in the DNA repair process. It will be important moving forward for us to understand how these drug-specific variations in DNA damage and repair relate to treatment at the clinical level.

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P42-39: GENES REGULATED BY THE HER2 RECEPTOR OF HUMAN BREAST CANCER CELLS

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Background: The HER2 member of the EGF receptor family of growth factor receptors is overexpressed in approximately 25% of breast cancer cases and confers a poor prognosis. Specific humanized monoclonal antibodies such as Herceptin[®] have provided an important therapeutic advance. Moreover, recent clinical trials indicate that synergistic therapeutic results may be obtained by combining Herceptin with cisplatin and other platinum-containing chemotherapeutic agents. However, this synergism is confined to HER2-expressing tumors. If the crucial downstream target(s) of Her2 signaling were known, it may be possible to target these genes in any other tumor type where they are recruited and, therefore, generalize the synergetic effect by combining cisplatin treatment with agents that block HER2 targets.

Methodologies: To this end, we have investigated a panel of human breast cancer cells with graded expression of HER2 and examined potential targets of regulation of HER2 by western analysis.

Results to Date: HER2 levels of either growing cells or quiescent breast cancer cells are not correlated with several signal transduction intermediates implicated in the literature, including activated Erk1/II, activated Erk5, and the transcription factors c-fos and Egr1, which are commonly rapidly induced by EGF or addition of serum. Further, the levels of these factors do not correlate with HER2 levels after addition of EGF or serum. However, the level of phosphorylated HER2 strongly correlated with the protein level of HER2, and this was also observed in serum-starved cells. In parallel with these findings, Akt was observed to be activated (phospho-Akt) in serum-starved cells in proportion to HER2 and phospho-HER2 levels. Finally, the transcription factor Elk-1 was observed to be phosphorylated upon addition of serum in proportion to the level of HER2. These results agree with reports that Elk-1 may be a transcription factor that mediates HER2 signaling. To test this possibility, we plan to carry out chromatin immunoprecipitation experiments using antibodies to Elk-1 and phospho-Elk-1 to determine that activation is associated with increased DNA binding. In addition, it is planned to extend the above studies by the use of Genentech Herceptin and HER2 monoclonal antibody as a criterion that Akt activation and Elk-1 activation are indeed downstream of HER2 signaling. The gene regulated by Elk-1 under the influence of HER2 will be identified by application of our chip-on-chip protocol.

Conclusions: There is an excellent correlation between the levels of phospho-Elk-1 and phospho-HER2, a known transcription factor downstream of pathways activated by erbB-gene family members. To test whether Elk-1 regulates gene expression determined by HER2, it will be necessary to correlate the extent of DNA binding by Elk-1 with HER2 levels and to determine whether specific inhibition of HER2 also blocks Elk-1-dependent gene transcription. We plan to apply chip-on-chip to identify the genes preferentially bound and activated by Elk-1 in HER2-expressing and nonexpressing control cells and test whether expression is HER2 dependent and functional in the transformed phenotype. Such genes are likely new and significant targets for the treatment of breast cancer and other non-HER2-expressing tumors.

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MAGNETIC RESONANCE IMAGING II

Poster Session P43

P43-1: COMPUTER-AIDED DIAGNOSIS OF BREAST CANCER USING DCE-MRI: PRE-CLINICAL EVALUATION ON TWO INDEPENDENT CLINICAL DATA SETS

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Background and Objectives: Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) is being increasingly used in breast cancer diagnosis. Interpretation of the large amount of four-dimensional data in a clinical MRI study is a burdensome and even challenging task for the radiologist. The overall objective of our research is to investigate an automatic and efficient computerized system for analysis of DCE-MRI images in the task of distinguishing between malignant and benign breast lesions. Our system includes breast lesion extent assessment, feature extraction, and classification of breast lesions in terms of an estimate of the probability of malignancy. The computerized image analysis system was evaluated with two independent clinical data sets.

Methods: Our analysis involved two breast MR databases totaling 302 biopsy-proven lesions. T1-weighted 3D spoiled gradient echo sequence was used to acquire one pre-contrast series and 5 postcontrast series with a 1 min temporal resolution. The first data set (77 malignant and 44 benign) was obtained with a 1.5T Siemens scanner. The second data set (97 malignant and 84 benign) was from a 1.5T GE scanner. In our computerized lesion characterization, the breast lesions initially undergo 3D segmentation by the computer. Then, characteristic kinetic curves are automatically identified. Image features are then automatically extracted to characterize the lesions as benign or malignant. Characteristic features include (1) kinetic features that quantify the uptake and washout characteristics of the contrast agent; (2) 3D texture features that quantify the uptake inhomogeneity within the lesion; and (3) 3D shape descriptors that quantify the irregularity of the tumor. The features are combined by a trained neural network model to obtain an estimate of the probability of malignancy. We evaluate the system by training and testing the model on two independent data sets. The benign/malignant classification performance of the system is assessed by receiver operating characteristic (ROC) analysis. The area under the ROC curve (AUC) is used as a performance figure of merit.

Results: When tested on the first data set with the model trained on the second data set, an AUC value of 0.91 (standard error 0.03) was obtained. When tested on the second data set with the model trained on the first data set, an AUC value of 0.85 (standard error 0.03) was obtained.

Conclusion: We developed an automatic computerized system for objective and efficient analysis of clinical breast MR data with high accuracy. The system could potentially aid radiologists in achieving an improved interpretation and workup of breast MR images in terms of efficiency, consistency, and accuracy.

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P43-2: CLINICALLY PRACTICAL MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY PROTOCOL FOR IMPROVED SPECIFICITY IN BREAST CANCER DIAGNOSIS

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Conventional mammography is known to have high false positive rate (60%–80%) in detection of breast malignancy, resulting in unnecessary biopsies. The increasingly popular dynamic contrast enhanced (DCE) magnetic resonance imaging (MRI) technique demonstrated high sensitivity (88%–100%) but rather variable specificity (37%–97%) in diagnosis of breast cancer. In this study, a combined MRI/MR spectroscopy (MRS) protocol including DCE MRI, 1H MRS, and perfusion MRI was used to examine patients with suspicious breast lesions. By correlating MR data with pathology results, we sought to determine if this clinically practical MRI/MRS protocol improves the specificity in detection of breast malignancy.

A total of 113 patients with positive mammography findings were recruited to participate in this MR study thus far. Biopsy was performed after but usually within a week of the MR examination. The MRI/MRS protocol was conducted with a 1.5 T MR scanner. For DCE MRI, 8 series of sagittal volumetric images of the whole breast with suspicious lesions were acquired with a temporal resolution of about 15 sec. Gadolinium-based contrast agent (0.1 mmol/kg dose) was delivered by intravenous (IV) injection at the start of the second series acquisition. Rapid contrast enhancement in lesions with signal intensity reaching plateau by the fourth series was defined as positive finding. Any enhancement with continuous rising of signal intensity through eight series or no enhancement was defined as negative finding. The study was discontinued for patients with negative findings. Patients with positive findings continued to undergo single

voxel 1H MRS and perfusion MRI examinations. The detection of an apparent choline (Cho) peak (signal-to-noise ratio > 2) at 3.23 ppm was defined as positive finding for the MRS study. Another IV injection of contrast agent (0.1 mmol/kg) was administered during perfusion MRI acquisition. The relative blood volume map was generated from the perfusion imaging data. The striking enhancement in the lesion area on the map compared to normal tissue area was defined as positive finding for the perfusion MRI study.

By correlation with the pathology results as the reference standards, there were no false negative findings from DCE MRI studies, showing 100% sensitivity of this method. The specificity of DCE MRI was 61%. With the addition of 1H MRS data, the specificity improved to 90%. With further addition of perfusion MRI results, the specificity improved to 100%. This study shows that while DCE MRI has very high sensitivity in diagnosis of breast cancer, its specificity is unsatisfactory. The MRI/MRS protocol of combined use of DCE MRI, 1H MRS and perfusion MRI substantially improves specificity and may help to reduce unnecessary biopsies following positive mammograms. With its technology easy for implementation at any imaging site and short scanning duration, this MRI/MRS protocol may have the potential to become the standard screening tool following positive mammographic findings.

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P43-3: A NOVEL MACROMOLECULAR CONTRAST AGENT BASED ON PARAMAGNETIC CHEMICAL EXCHANGE SATURATION TRANSFER (PARACEST) FOR BREAST CANCER STUDIES

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Introduction: Although conventional small molecular MRI contrast agents have high sensitivity for breast cancer detection, they have limited specificity to characterize the detected lesions. Conventional macromolecular contrast agents have high specificity but the sensitivity is low. We propose to use a novel macromolecular agent, which belongs to the class paramagnetic contrast agents based on the chemical exchange saturation transfer (CEST) effect. Recently, an albumin-binding PARACEST agent was developed by us where the hydrophobic O-benzyl (OBz) groups act as HSA binding sites for the resulting lanthanide complexes. The albumin-binding agent TmDOTA-(Gly)2(OBz-Ser)2 is used here to study PARACEST contrast kinetics in rat breast tumors.

Methods: A spin-echo imaging sequence preceded by a WALTZ-16 preparation pulse train on the free water resonance was used to image concentration dependence of contrast in phantoms and contrast kinetics in vivo due to the PARACEST agent (i.e., Dynamic PARACEST Contrast Enhancement, DPCE). The MR parameters include a TR/TE=1,000/15ms, FOV = 40 mm x 40 mm, 128 x 128 matrix, and a 75 ms WALTZ16 train. The signal intensity I due to an on-resonance WALTZ-16 pulse train of total duration t_w is related to the concentration c of the agent by $I(c) = I(0) \exp(-c k_{eff} t_w)$ (eqn. 1), where $k_{eff} = (\tau_b/111) * (2 * \tau_b + T_{1b}) / (t_b + T_{1b})$, where τ_b is the bound water residence time, T_{1b} is the bound water T_1 , $I(0)$ is the intensity corresponding to the presence of a far off-resonance WALTZ pulse, and tissue water concentration is assumed to be 55.5M.

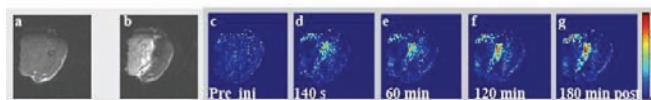


Figure: a) T1 and b) T2 weighted images of a MTLN2 rat breast tumor implanted on the thigh.

Results and Discussion: In phantom studies, albumin-binding PARACEST agent, TmDOTA-(Gly)2(OBz-Ser)2, displays a small but significant (~30% at 0.75 mM) relative CEST contrast enhancement. In the presence of 0.75 mM HSA in PBS, a smaller relative enhancement is seen (~20% at 0.75 mM) when the complex binds with HSA and is likely due to a small increase (~5%) in the bound water residence time and shorter T_2 of the HSA solution. Post injection of 0.1 mmole/kg TmDOTA-(OBz-Ser)2-Gly2 in MTLN2 tumor-bearing rats, an early enhancement is seen followed by a slow increase in contrast enhancement over 3 hrs corresponding to pooling of the albumin-bound agent. TmDOTA-(Gly)2(OBz-Ser)2 is shown to be a promising albumin-binding agent for use as a macromolecular PARACEST agent in vivo for breast cancer studies.

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P43-4: METABOLITIC CHARACTERIZATION OF BREAST CANCER IN VIVO USING TWO-DIMENSIONAL MR SPECTROSCOPY

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Background and Objectives: Magnetic resonance spectroscopy (MRS) allows noninvasive measurements of metabolites. MRS of human breast tissues shows the resonances due to water, choline groups, nucleotides, and saturated and unsaturated fatty acids. Previous research has shown that the water/fat ratios are high, and a choline (CHO) peak appears in malignant breast tissues (1, 2). A major objective of this work is to analyze the metabolite ratios in different breast tissues to differentiate between malignant and healthy tissues using two-dimensional (2D) localized correlated spectroscopy (L-COSY).

Methods: A total of 8 women with invasive ductal carcinomas (mean age 51 years old) and 8 healthy women (mean age 43 years old) have been investigated. All scans were performed on a 1.5T Avanto whole body MRI/MRS scanner with a dedicated Siemens phased array breast coil (Siemens Medical Systems, Erlange, Germany). 2D L-COSY spectra were acquired from the localized region of interest (ROI), typically 1 ml, in malignant, healthy fatty, and glandular breast tissues. The raw data were processed using the FELIX software (Felix NMR Inc., San Diego, California).

Results to Date: 2D L-COSY spectra can detect 10 different spectral peaks indicated by parts per million (ppm) locations: (1) Water (WAT) at (4.8,4.8) ppm; (2) Fat (FAT) at (1.4,1.4) ppm; (3) Choline (CHO) at (3.3,3.3) ppm; (4) Methyl Fat (FMETD) at (0.9,0.9) ppm; (5) Unsaturated fatty acid cross peaks right (UFR) at (2.1,5.4) ppm; (6) Unsaturated fatty acid cross peak left (UFL) at (2.9,5.4) ppm; (7) Olefinic Fat (UFD) at (5.4,5.4) ppm; (8) Triglyceryl fat cross peak (TGFR) at (4.3,5.3) ppm; (9) FAT2 at (2.1,2.1) ppm; and (10) FAT3 at (2.9,2.9) ppm. Different metabolite ratios in malignant, healthy fatty and healthy glandular breast tissues are presented as (Mean±Standard Deviation):

WAT/FAT:8.30±2.35;0.024 ±0.014;1.468 ±0.614;WAT/CHO:613±156;32.3±9.7;899.1 ±335;WAT/FMETD:78.8±28;0.368±0.206;32.0±23.8;

CHO/FAT:0.014±0.01;0.00062±0.00006;0.0018±0.0038;CHO/FMETD:0.134±0.074;0.0084±0.0011;0.014±0.0033.

We use the two cross peak ratios UFL/UFR as the indicator degree of unsaturation and the calculated ratio (UFL/UFR) in invasive carcinoma, healthy fatty, and glandular tissues as: 0.941 ± 0.149; 0.681 ± 0.10; 0.997 ± 0.117.

Discussions and Conclusions: From the above listed metabolite and lipid ratios, we can conclude that metabolite ratios are different in different breast tissues; UFL/UFR is different in malignant, glandular, and healthy fatty tissues. The measurement of "degree of unsaturation" in breast lipids may be used as a potential biomarker to differentiate between malignant and healthy tissues. Investigation in a larger patient cohort is required to further validate the current findings.

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P43-5: AN ENZYME-RESPONSIVE PARACEST MRI CONTRAST AGENT THAT DETECTS CATHEPSIN B IN METASTATIC BREAST TUMORS

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Background: Cathepsin B promotes breast tumor metastasis [1] and is an exoprotease at low pH in tumor tissues. A new type of MRI contrast agent can be detected through the PARAMagnetic Chemical Exchange Saturation Transfer (PARACEST) effect that originates from an amide [2]. PARACEST MRI contrast agents can be designed to be cleaved by an exoprotease, which converts the amide to an amine and alters the PARACEST effect [3,4]. This investigation will develop a PARACEST MRI contrast agent that can detect Cathepsin B within an MDA-MB-231 subcutaneous tumor mouse model (Figure 1A).

Methodologies: Customized solid-phase peptide synthesis methods [5] were used to create a CBZ-Arg-Arg-(Yb-DOTA) MRI contrast agent. A molecular model of the Cathepsin-B:CBZ-Arg-Arg-(Yb-DOTA) complex was constructed using Insight II (Molecular Simulations, Inc.) [6]. The Michaelis-Menten kinetics of exoprotease cleavage of the PARACEST agent will be measured, and a PARACEST agent that does not interact with Cathepsin B, Eu-DOTAMGly, will be included as a control. To conduct

in vivo tests, the enzyme-responsive and unresponsive agents will be detected in an MDA-MB-231 subcutaneous flank tumor mouse model. The tumor concentration of Cathepsin B will be validated by using immunohistochemistry.

Results: The CBZ-Arg-Arg-(Yb-DOTA) PARACEST contrast agent was synthesized and demonstrated a PARACEST effect ranging from -16 ppm to -25 ppm (Figure 1B). The molecular model verified that the PARACEST contrast agent can be accommodated within the active site of Cathepsin B and with favorable hydrophobic and hydrophilic interactions between the agent and protease (Figure 1C). Michaelis-Menten kinetics studies and in vivo PARACEST MRI studies are in progress.

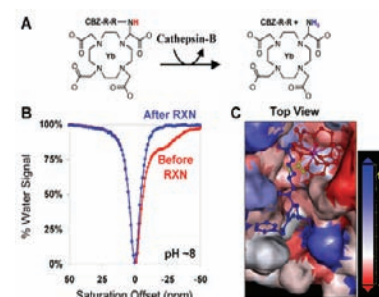


Figure 1. (A) protease reaction
 (B) CEST spectra
 (C) model of cathepsin-B:agent complex

Conclusions: Detection of Cathepsin B activity in breast tumors may aid in diagnosing metastasis. This research also demonstrates the capabilities of this platform technology to diagnose other breast cancer protease biomarkers.

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P43-6: DEVELOPMENT OF CELL-TYPE-SPECIFIC CONTRAST AGENTS FOR MAGNETIC RESONANCE IMAGING OF BREAST TUMORS

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Physical Sciences, Inc.

Background and Objectives: Magnetic resonance imaging (MRI) is an emerging diagnostic tool for breast cancer management. This powerful technique provides a high-resolution three-dimensional image portraying morphological features of tissues and organs. However, the anatomic details delineated even at very high resolution are not sufficient for diagnosis of malignant lesions as they are often indistinguishable from normal and benign structures. Increase in detection specificity of cancerous lesions is achieved using contrast agents (CA). The current approach for targeting of tumor-specific CA is based on conjugation of non-specific CA with antibodies (Abs) or fragments thereof. The major disadvantages of Ab-mediated targeting are the large size of Ab molecules and their ability to induce an unwanted immune response in a patient. An alternative approach for CA targeting is the utilization of aptamers, synthetic single-stranded DNA molecules that bind to target molecules with high affinity and specificity. Aptamers are much smaller than Abs yet display comparable binding properties. Aptamers have been demonstrated in animal studies to be non-toxic and non-immunogenic, features important for use in humans. The objective of this research effort was to test our novel concept of using DNA aptamers for targeting MRI CA to the surface of breast cancer cells.

Brief Description of Methodologies: Extracellular domain (ED) of the HER2/Neu (Erb-B2) receptor was chosen as a target for selection of DNA aptamers. Systematic evolution of ligands by exponential enrichment (SELEX) was used for isolation of aptamers specific against ED of Erb-B2.

Results to Date: We prepared DNA expression construct encoding for the entire ED of human Erb-B2 which was modified by an epitope tag for antibody detection and histidine tag for protein purification. This construct was used for generation of stable cell line expressing Erb-B2 fusion protein. We confirmed that the construct was efficiently expressed and properly targeted to the cell membrane. For the aptamer selection procedure, partially purified membranes of the cells expressing Erb-B2 were extracted with mild detergent and the resulting membrane extract was combined with combinatorial synthetic library of oligonucleotides containing a 40 base long variable region. Erb-B2 was isolated using metal chelating chromatography followed by an immunoprecipitation (IP). The aptamers amplified from Erb-B2/aptamer complexes after the IP were used or the next round of purification as above. We have performed 4 subsequent rounds of selection after which amplified aptamers were cloned and sequenced. A group of 18 sequences displaying significant similarity were identified that could be indicative of efficient aptamer enrichment. Testing of these aptamers in Erb-B2 binding assays was not carried out under this program but will be performed in the near future.

Conclusions: In the course of the research effort we have generated valuable reagents including DNA expression constructs, characterized stable cell lines, and identified candidate aptamer sequences. This accomplishes the first step essential for generation of aptamer-targeted MRI CA. Our research group will continue this project using generated reagents and expertise and will prepare Erb-B2 specific CA. It is anticipated that the specificity and the enhanced tissue penetration of such MRI CA will benefit breast cancer patients by improving early detection of the disease.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0769.

P43-7: MULTIMODAL IMAGING OF BREAST CANCER IN GENETICALLY ENGINEERED MOUSE MODELS

Minghua Xu, Lewis A. Chodosh, Alexander J. Stoddard, Kathleen Notarfrancesco, Katherine D. Dugan, George K. Belka, Celina D'Cruz, Lin Li, Valentina Circumaru, Stephen Pickup, Mitchell D. Schnall, Abass Alavi, Joel Karp, and Paul Acton
University of Pennsylvania

Breast cancer is the leading cause of death among women in Western countries, but the mechanisms of human breast cancer progression are largely unknown. Understanding the molecular, cellular, and pathophysiological events that contribute to this process is therefore essential. Our laboratory has previously developed a novel series of bitransgenic mouse lines that mimic the natural course of breast cancer development in humans, including the development of resistance to therapy, metastasis, and recurrence. These mouse lines permit oncogenes relevant to human breast cancer to be turned "on" and "off" in mammary glands of mice in response to doxycycline administered in their drinking water. However, the need to sacrifice the animals to perform tissue or molecular analysis prevents researchers from observing in vivo the natural or perturbed evolution of the processes under study.

The objective of this project is to employ multimodal in vivo cellular and molecular imaging approaches to analyze genetically engineered mouse models of breast cancers for understanding the mechanisms of breast cancer progression. Imaging approaches including magnetic resonance imaging (MRI), positron emission tomography (PET), X-ray computed tomography, and other potential methods are used to assess key parameters of tumor biology such as cellular proliferation, tumor perfusion, vascular permeability, tumor blood flow, tumor oxygenation, and tumor glucose utilization.

First, we have used diffusion MRI to detect mammary tumor early response to oncogene downregulation with doxycycline withdrawal in transgenic mouse models. MRI studies showed a statistically significant increase of apparent diffusion coefficient values in solid mammary tumors within 48 hours after oncogene deinduction, accompanied by slight decreases of T2 value and tumor size. This result demonstrates that diffusion MRI analysis provides an early biomarker for mammary tumor response to oncogene downregulation.

Second, we have used PET to evaluate differential uptake of ^{18}F -FDG in primary mammary tumors that inducibly express various human oncogenes. FDG-PET imaging studies demonstrated that FDG uptake varied substantially among primary mammary tumors with the expression of different oncogenes although these tumors shared similar growth curves. Comparison of FDG uptake with microarray analysis for glucose metabolism indicated that there were strong correlations of FDG uptake with hexokinase 1 expression in primary mammary tumors. Comparison of FDG uptake with immunohistochemical tissue analysis for biological characteristics failed to show a universal correlation between FDG uptake and cell proliferation rates measured by BrdU incorporation and also failed to show a strong correlation between FDG uptake and apoptosis measured by TUNEL studies. These results indicate that specific oncogenic pathway activated determines tumor characteristics, including the degree of glucose metabolism and the subsequent variation of FDG uptake in breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0523.

P43-8: COMBINED ANATOMICAL AND BIOCHEMICAL CHARACTERIZATION OF BREAST CANCER

Michael Albert Thomas,¹ Scott Lipnick,² Xiaoyu Liu,³ Nanette Debruhl,² and Lawrence Bassett²

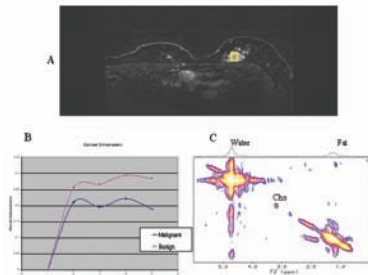
¹University of California, Los Angeles, David Geffen School of Medicine, ²University of California, Los Angeles, and ³Imperial College, London, UK

Background and Objectives: Currently, dynamic contrast enhanced (DCE) magnetic resonance imaging (MRI) is the most sensitive imaging modality for the detection of breast cancer. A drawback, however, is that the reported specificity of DCE-MRI varies between 37% and 97%, resulting in many unnecessary biopsies of benign lesions (1). Studies of breast cancer cell lines and breast tumors have consistently shown that choline (Cho) is elevated in malignant masses using MR spectroscopy (MRS) (2-3). The purpose of this study was to investigate if localized two-dimensional correlated

spectroscopy (2D L-COSY) combined with DCE-MRI will improve the overall specificity in discriminating malignant from the benign breast tumors and healthy breast tissues.

Methods: We have recruited a total of 10 women with invasive ductal carcinomas (mean age 51 years old), 3 women with benign lesions (mean age 30 years old), and 13 healthy women (mean age 43 years old) so far. The DCE-MRI (1,3) and 2D L-COSY (4) scans were performed on a 1.5T Avanto whole body MRI/MRS scanner using a dedicated phased-array breast coil for data acquisition. Due to technical failures involving shimming of the main magnetic field (B₀), two data sets were not used in the final analysis.

Results to Date: Figure 1A shows a DCE-MRI of a 56-year old woman with invasive carcinoma marked with the volume localized for 2D L-COSY. The enhancement curves from the discussed malignant lesion and a typical benign lesion are shown in Figure 1B with the associated 2D L-COSY spectrum in Figure 1C. In Figure 1B, both malignant and benign lesions showed type II enhancement curves and were indistinguishable using DCE-MRI alone. However, the associated 2D spectra from the malignant lesions showed a choline peak while the benign did not. The sensitivity was 90% and specificity 100% from our pilot results. The lesions that would have shown previously uncertain results (type 2=plateau enhancement) now can be differentiated through the combination of DCE-MRI and 2D L-COSY.



Conclusions: The sensitivity of DCE-MRI was 90% and that inclusion of 2D L-COSY increased the specificity to 100%. This is in agreement with previous studies that have shown choline can be used as a clinical marker for malignancy.

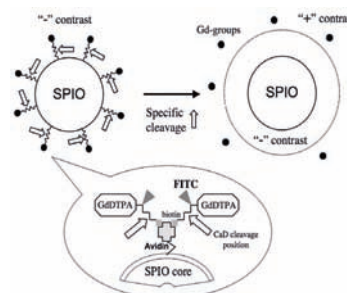
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This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0565.

P43-9: MOLECULAR MR IMAGING OF PROTEASE ACTIVITY IN BREAST CANCER WITH ACTIVATED CONTRAST AGENTS

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Background/Hypothesis: We have recently developed an activated contrast agent for magnetic resonance imaging (MRI) that contains both superparamagnetic iron oxide (SPIO)-negative contrast agent and low molecular weight GdDTPA-positive contrast agent, using a novel technology called a dual-contrast technique. In this project, we focus on Cathepsin-D, which is a proteolytic enzyme overexpressed in breast cancer cells, and MR contrast agent can be activated by this enzyme in acidic extracellular tumor microenvironment. The intact molecule produces strong negative T2 contrast in T1/T2-weighted MRI due to high T2 relaxivity of SPIO particles. Upon cleavage of Cathepsin-D-specific linkers, multiple Gd groups diffuse away from the SPIO core and generate positive T1 MR contrast on T1-weighted images (Figure 1). At least 5 times longer diffusion range in tissue is expected for ~2 nm GdDTPA molecules in comparison to ~50 nm SPIOs diffusion of which is significantly restricted in vivo. Therefore, imaging probes activated by a specific tumor-associated enzyme can significantly improve tumor/background ratio.



Schematics of the agent structure and activation process

Materials and Methods: To confirm our concept of dual MR contrast technique and to simulate diffusion properties of the tissue, we constructed a liposome model system for in vitro MRI experiments using 2% agarose gel systems. MRI experiments were performed with a horizontal bore Bruker Biospec 9.4T small animal MR scanner. Standard T1/T2 spin-echo imaging sequences were used for acquisition. The Paravision 3.0.2 program (Bruker Biospin GmbH) was used as an acquisition software. Quantitative T1 maps of the samples were reconstructed using custom-written IDL software. Final analysis was performed with the ImageJ(R) program (NIH). A Cathepsin-D-degradable peptide linker is currently being synthesized using dedicated automatic peptide synthesis facilities. Peptide chain with a structure: Lys(Biotin)-Gly-Pro-Ile-Cys(Et)-Phe-Phe-Arg-Leu-Gly-Lys(FITC)-Gly-Lys(GdDTPA) is grown on amide resin using N-(9-fluorenyl)methoxycarbonyl (N- α -Fmoc)-protected amino acids (italicized amino acids correspond to Cathepsin-D cleavage site). Selective deprotection of the side chains amino group is used for orthogonal modification of the peptide.

Results/Conclusions: When sequestered with the same carrier molecule, the negative T2 effect of SPIO nanoparticles dominates the positive T1 contrast produced by GdDTPA. Released GdDTPA small molecular weight groups diffuse from the carrier and generate positive contrast in MR images by reducing T1 values of the sample as confirmed in in vitro agarose gel system. Emergence of the positive contrast by decomposition of the dual MR contrast agents-loaded carrier using a liposome model system supports our concept behind activated MR contrast agent. These findings will facilitate noninvasive MR imaging of breast cancers overexpressing Cathepsin-D that is linked to tumor invasion, angiogenesis, and metastasis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0610.

NANOTECHNOLOGY II

Poster Session P44

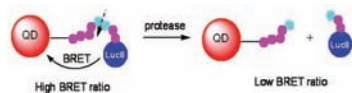
P44-1: QUANTUM DOT-BASED NANOSENSORS FOR MATRIX METALLOPROTEINASE DETECTION AND IMAGING

Jianghong Rao, Zuyong Xia, Min-Kyung So, and Yun Xing
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Matrix metalloproteinases (MMPs) are a family of secreted endopeptidases that play a crucial role in defining the cellular environment through regulated degradation and processing of extracellular matrices. MMPs are upregulated in almost every type of human cancer including breast cancer. MMP overexpression correlates with advanced tumor stage, increased invasion and metastasis, and shortened survival. To better dissect the interplay of MMPs during tumor development and invasion, a sensitive assay that can detect multiple MMPs simultaneously would be of great value, but current methods including zymographic and fluorometric assays cannot do so.

In this work, we explored a novel and general strategy based on semiconductor nanocrystals (quantum dots, or QDs) and the principle of bioluminescence resonance energy transfer (BRET), which is an energy transfer phenomenon between a light-emitting protein and a fluorescent acceptor; in short, we called it QD-BRET. We have discovered that when a mutant of the bioluminescent protein *Renilla luciferase* (Luc8) was coupled to the QDs, the biochemical energy generated by Luc8 in the oxidation of the substrate coelenterazine can be transferred to QDs nonradiatively and produces the QD light emission (*Nature Biotechnology* 2006, 24, 339).

Based on this QD-BRET technology, we designed a nanosensing system where Luc8 is linked to the QD through an MMP-cleavable peptide substrate (Scheme 1). In the absence of the target MMP, the QD conjugate can produce QD emission due to the BRET from Luc8. When the MMP is present, the peptide linker will be cleaved, leading to the releasing of the Luc8 from the QD and thus the disruption of the BRET. An intein-mediated protein splicing chemistry was applied to ligate the fusion of the peptide substrate and the Luc8. This method allows site-specific conjugation of the fusion protein to QDs so the MMP hydrolysis can cleave the Luc8 from the QDs.



Scheme 1. Detection of MMP activity with a QD-BRET nanosensor

Using this method, we have prepared several QD-BRET sensors for MMP-2, MMP-7, and urokinase detection. We were able to detect these proteases in buffer, tumor lysates, and in mouse serum. Multiplexed detection of several proteases using QD-BRET probes has also been achieved.

In conclusion, we have successfully designed the QD-BRET nanosensors for sensing MMPs and shown that these nanosensors are able to detect MMPs at high sensitivity in biological samples. We envision that the detection system would find potential wide applications such as in real-time monitoring of MMP activity in clinic samples and in facilitating the screening of inhibitors for MMPs as novel breast cancer therapeutics.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0642 and National Institutes of Health.

P44-2: MAGNETIC NANOPARTICLE-BASED DETECTION OF RNA TRANSCRIPTS

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According to the American Cancer Society, breast cancer is the second leading cause of cancer deaths in women today. In 2005, it was estimated that 211,240 new cases of invasive breast cancer would be diagnosed among women, and approximately 40,410 women were expected to die from the disease. Clearly, from these staggering numbers there remains an urgent need for the development of novel methods to better define breast cancer risk and guide therapy more rationally. One promising approach is to noninvasively identify early markers of breast cancer using molecular imaging agents. Survival rates for breast cancer have been strongly linked to early detection, and evidence suggests that finding breast cancer early can improve the likelihood that treatment will be successful. Since it is generally understood that cancers arise from the gradual accumulation of genetic changes, it is expected that the identification of changes in gene expression would provide the earliest opportunity to detect malignancies. Recently, we developed a novel approach to detect RNA transcripts via magnetic resonance by taking advantage of the decrease in the spin-spin (i.e., T_2) relaxation time that results from the self-assembly of superparamagnetic iron oxide nanoparticles (NPs). Specifically, two unique NP-oligonucleotide conjugates were designed to recognize adjacent sites on nucleic acid targets. Thus, upon hybridization to complementary targets, the NP-oligonucleotide conjugate pairs were brought into close proximity, which resulted in a detectable reduction in the T_2 relaxation time. This mechanism of switching from a high T_2 -relaxation time to a low T_2 -relaxation time during NP self-assembly (or vice versa during NP disassembly) is generally referred to as magnetic relaxation switching (MRSW). In the presence of target nucleic acids, we have measured as much as a 40% decrease in T_2 signal due to aggregate formation with reliable detection of target at levels as low as 10 pmoles. Furthermore, we have observed that the detection range and sensitivity of the MRSW depends upon the configuration (iron oxide core

size, relaxivity, and ligands per NP) of the NPs involved in the switch. Currently, we are working to optimize the lower detection limit of our technology, and we are evaluating various methods to efficiently deliver our NPs into the cytoplasm of breast cancer cells where they will have direct access to intracellular mRNA. We envision that the ability to image mRNA transcripts within cancer cells could have a major impact on the early detection, staging, and treatment of breast cancer. The identification of changes at the genetic level would provide the earliest opportunity to detect malignancies. Further, insight into the actual mechanism responsible for aberrant cell behavior would allow clinicians to more accurately identify the stage of cancer progression and would provide them with the information necessary to guide therapy more rationally. Our mRNA-sensing technology could also be used for diagnostic assays or potentially be used to aid in the development of therapeutics (e.g., gene therapy and siRNA).

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0457.

P44-3: DOUBLE-HIT MOLECULAR THERAPY FOR erbB2(+) BREAST CANCER BY THE SELF-ASSEMBLED HERCEPTIN®-NANOVECTOR-MIRNA INHIBITS BREAST CANCER STEM CELLS

Min Zhang, Qing Ji, Theodore Lawrence, and Liang Xu
University of Michigan

miRNAs are a conserved class of noncoding RNAs that regulate gene expression post-transcriptionally. miRNAs regulate a variety of biological processes, including developmental timing, signal transduction, tissue differentiation and maintenance, disease, and carcinogenesis. Emerging evidence demonstrates that miRNAs also play an essential role in stem cell self-renewal and differentiation by negatively regulating the expression of certain key genes in stem cells. Our study shows that miR-34 potently suppresses Notch, MET, HMGA and Bcl-2, genes involved in cancer stem cell self-renewal and survival, and inhibits breast cancer mammosphere growth and tumor formation, indicating that tumor suppressor miRNAs such as miR-34 may hold significant potential as a novel molecular therapy for cancer. However, delivering the miRNA-based therapeutics efficiently and specifically to tumor and its metastases remains a great challenge.

With the support of a DOD BCRP Concept Award, we have developed a self-assembled nanoparticle system targeted by anti-erbB2 antibody, Herceptin®. This system is based on our patented nanovector technology that shows promising efficiency in targeted delivery of p53 gene and Ras/Her-2-anti-sense oligonucleotides to breast tumors (U.S. Patent No. 6,749,863) and has been approved by FDA and now is in Phase 1 clinical trial. These self-assembled nanovectors have a novel nanostructure that resembles a virus particle with a dense core enveloped by a membrane coated with targeting molecules spiking on the surface.

Recent clinical study using the erbB2 kinase inhibitor, Tykerb, showed promising clinical efficacy with long-term survival in erbB2(+) breast cancer patients, concomitant with the reduction of breast cancer stem cells, linking the erbB2-targeted therapy and its efficacy to breast cancer stem cells. In the current study, we established and optimized the Herceptin-nanovector-miRNA for tumor-targeted delivery of miRNAs to erbB2(+) breast cancer in vitro and in vivo. Our hypothesis is that Herceptin and miRNA double therapy by the same nanoparticle (double-hit) may achieve better efficacy by inhibiting breast cancer including breast cancer stem cells. Two erbB2(+) breast cancer cell lines, SKBR3 and MCF-7-Her2-H18, were chosen as targeting cell lines. Using β -Galactosidase gene reporter assay, we found that with the optimal formulation, the transfection efficiency was 2-fold of the normal IgG control nanovectors in both cell lines. Then we employ Herceptin-nanovectors to encapsulate miR-34a and miR-16; both have been reported to be potential tumor suppressors and involved in the regulation of stem cell-like properties in our hands. Treatment of Herceptin-nanovector-miRNA significantly reduced both the number of mammospheres and the cell number per mammosphere as compared with normal IgG-nanovector-miRNA or control miRNA. Colony formation of the treated erbB2(+) cells was also inhibited. Tumor initiation experiments and breast cancer stem cell analysis are still ongoing.

Taken together, the double-hit molecular therapy by the self-assembled Herceptin-nanovector-miRNA shows promise as a novel treatment for erbB2(+) breast cancer via inhibiting breast cancer stem cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0648 and National Cancer Institute.

P44-4: MR IMAGING OF ULTRASENSITIVE, BREAST CANCER TARGETING SUPERPARAMAGNETIC POLYMERIC MICELLES IN VIVO

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University of Texas Southwestern Medical Center at Dallas

Magnetic resonance imaging (MRI) is a major imaging modality in cancer diagnosis due to its superb in vivo imaging capability with high resolution, excellent soft tissue contrast, and sensitivity to blood flow. Currently, much effort is devoted to the detec-

tion of cancer molecular markers by MRI using paramagnetic (e.g., Gd-DTPA) and superparamagnetic agents (e.g., Fe_3O_4). However, the primary limitation of these agents is their lower sensitivity of detection. Here we report the development of superparamagnetic polymeric micelles (SPPM) as ultrasensitive, cancer-specific MRI probes for in vivo imaging of breast cancer.

SPPM were prepared by encapsulating Fe_3O_4 nanoparticles inside the core of poly(ethylene glycol)-poly(D,L-lactide) (MW=10kD) polymeric micelles. cRGD peptide was conjugated to the shell of the SPPM at 50% surface density. SPPM were characterized by dynamic light scattering (DLS) and transmission electron microscope (TEM). In vitro cell uptake was performed in SLK tumor endothelial cells. Athymic nude mice bearing subcutaneous MDA-MB-231 breast cancer xenografts were injected i.v. with SPPM at 20 mg Fe/kg dose. T_2 -weighted images of the mouse pre- and postinjection were obtained using a 4.7 T Varian small animal imager.

TEM and DLS analyses revealed that SPPM had an average diameter of 70 ± 14 nm. T_2 -weighted MR images of phantoms containing SPPM solutions showed significant signal loss when the SPPM concentration increased. SPPM conjugated with cRGD showed greater uptake in SLK endothelial cells than non-cRGD-SPPM because cRGD ligands bind to $\alpha_v\beta_3$ integrin overexpressed on the surface of SLK cells inducing endocytosis. Mice bearing subcutaneous MDA-MB-231 breast tumors were injected with SPPM (with and without cRGD) at 20 mg Fe/kg dose. By comparing pre- and postinjection images, the tumor of the mouse injected with cRGD-SPPM showed significant T_2 contrast as a dark region inside the tumor due to the accumulation of SPPM inside the tumor. However, the tumor of the mouse injected with non-cRGD-SPPM did not contain any dark region. In addition, T_2 relaxation times of the tumor in the cRGD mouse were greatly shortened after the injection (from 56 ms to 35 ms) while those of the tumor from mouse injected with non-cRGD-SPPM remained unchanged.

Here we demonstrate the development of SPPM with MRI ultrasensitivity and cancer specificity. The SPPM can be specifically delivered to the tumor tissues and produced considerably increased MR contrast. This class of new contrast agents with imaging ultrasensitivity and cancer specificity shows promising potential toward MRI molecular imaging in breast cancer detection.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0751.

P44-5: ULTRASENSITIVE MULTIMODALITY IN VIVO IMAGING USING NANOPARTICLES

Fanqing Chen,¹ Julie Herberg,² Daniele Gerion,³ Louis Bouchard,³ Sabieh Anwar,³ Byron Hann,⁴ Gang L. Liu,² Harry Z. Xie,⁵ Robert Bok,⁴ Erica Gjersing,² John Kurhanewicz,⁴ Xueding Wang,⁶ Alex Pines,³ and Joe Gray¹

¹Lawrence Berkeley National Laboratory, ²Lawrence Livermore National Laboratory, ³University of California, Berkeley, ⁴University of California, San Francisco, ⁵Bruker Optics, Inc., and ⁶University of Michigan

In situ detection and imaging of breast cancer at an early stage are vital for clinical intervention and patient survival. Conventional mammogram has very high false-positive rates. Recently, antibodies specific to breast tumor cells have gained popularity in early diagnosis. However, conventional imaging modalities using conventional contrasting and reporter agents have limited sensitivity and lack long-term optical and chemical stability, making diagnostic molecular imaging very difficult to implement.

Multimodality imaging with different and complementary detection principles can greatly improve the accuracy and sensitivity of diagnosis and has a broad range of clinical implications. Incorporation of multiple modalities into a single delivery vehicle has been a bottleneck until recent advances in nanoparticle bioengineering in which multiple functional components can be integrated into a single physical unit. Funded by the BCRP, we present here two cutting-edge nanoparticles developed for multimodality imaging: Paramagnetic optical nanoparticle and superparamagnetic Au nanoparticle that can be imaged with photoacoustics and MRI. Both allowed us to achieve sensitivity unparalleled, even approaching sensitivity of radioisotope labeling in vivo.

The first nanoparticle composite is a novel nanocomposite of paramagnetically coated quantum dot (Qdot) that can be detected at nanomolar range by NMR and has strong near-infrared (NIR) fluorescence emission that penetrates deep tissue to allow for NIR optical imaging. In magnetic resonance imaging (MRI) experiments at clinical magnetic field strengths of 1.4 Tesla (^1H resonance frequency of 60 MHz), the gadolinium-DOTA (Gd-DOTA) attached to SiO_2 coated QD has a spin-lattice (T_1) particle relaxiv-

ity (r_1) and a spin-spin (T_2) particle relaxivity (r_2) of $1019 \pm 19 \text{ mM}^{-1}\text{s}^{-1}$ and $2438 \pm 46 \text{ mM}^{-1}\text{s}^{-1}$, respectively. The sensitivity of our probes is in the 100 nM range for 8–10 nm particles and reaches 10 nM for particles with approximately 15 nm in diameter.

The second nanoparticle composite is based on a nanowonton scheme. In recent years, zero valence ferromagnetic particles, such as cobalt, iron, or nickel, and their derived alloys, have emerged as attractive contrast agents for magnetic resonance imaging. One major unsolved issue is the relatively high reactivity and instability of these ferromagnetic materials. Here we provide a very convenient general physical method for fabricating Co nanoparticles using highly stable thin film protective gold (Au) coating. Furthermore, we have engineered photoacoustic tomography (PAT) imaging modality into the nanoconstruct by introducing shape-dependent tuned light absorbance property. The end result is a highly sensitive dual modality nanoprobe whose accompanying image contrast can be detected in vivo at picomolar concentrations. Therefore, these particles are orders of magnitude more sensitive than superparamagnetic MRI particles even in the complex in vivo environments and approach the sensitivities of radiolabels.

We have also successfully developed a surface chemistry modification scheme that can encapsulate the Qdots in a silica shell with minimum in vivo toxicity. This endeavor could lead to an ultrasensitive and quantitative solution of early diagnosis and treatment efficacy monitoring with human translational potential.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0525.

P44-6: LUMINESCENT ORGANIC NANOPARTICLES FOR BREAST CANCER DETECTION AND IMAGING

Timothy M. Swager

Massachusetts Institute of Technology

In this contribution, we will detail the synthesis of luminescent organic nanoparticles with high two-photon absorption cross-sections. The chemistries for formation of the nanoparticles will be detailed, and the structure property relationships that give rise to that large two-photon absorption will be discussed. The nanoparticles will be multi-component and will down-convert the initial excitations through energy transfer to a long wavelength luminescence in regions where human tissue is relatively transparent. The nanoparticles will be readily conjugated with ligands that can bind to cancer cells, and in this way, we expect to be able to develop early-stage detection methods for breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0649.

P44-7: MULTIFUNCTIONAL APPLICATIONS OF GOLD-PHAGE NANOSHUTTLES

Glauco Souza, Wadih Arap, Wadih Arap, Renata Pasqualini, and Renata Pasqualini

M. D. Anderson Cancer Center, University of Texas

As we enter an age of engineering molecular assemblies, the combination of nanotechnology, phage display, and biophotonics offers remarkable opportunities to improve the diagnosis and treatment of cancer. It is not trivial to design and implement an entity with nanodimensions that can reproducibly and effectively integrate different functions, such as signal reporting, drug targeting, drug delivery, as well as have therapeutic properties. The concept being developed in this work combines phage display technology and gold nanoparticles (Au) in a "bottom-up" molecular direct-assembly of Au and phage into the nanoshuttle structures (Au-phage nanoshuttles). The Au-phage nanoshuttles uniquely combine different properties, including the capability of converting near infrared (NIR) radiation into heat, providing signal enhancement for fluorescent imaging and surface enhanced Raman scattering (SERS) detection, and functioning as

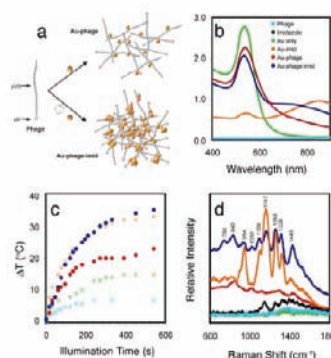


Fig. 1. (a) Strategy for Au assembly onto phage nanoparticles (nanoshuttles). Imidazole and the yellow spheres (Au nanoparticles; not drawn to scale; the gold particles have a diameter of 44 ± 9 nm, and the pVIII capsid peptide a thickness of ~ 8 nm). (b) Light absorption spectrum of purified and suspended Au-phage-imid (dark blue), Au-phage (red). (c) Temperature increase (785 nm laser light) time of Au-phage-imid (blue) and Au-phage (red) solutions; the controls (open circles) are the solutions of Au (green), Au-imid (orange) and phage (cyan). The concentrations of solutions carrying Au were normalized according to the area under the absorption region of the spectra (above 475 nm). Solution temperature was measured with a digital temperature probe immersed in 300 μl of solution and 5 mm away from the laser focal point. (d) SERS of Au-phage-imid (blue) and Au-phage (red). (e) SERS spectra for each nanoshuttle.

contrast agents for magnetic resonance imaging (MRI, with FeO in nanoshuttle) and computed tomography (CT) scanning. The objective of this work is to exploit the multimodal imaging properties and the programmable targeting capabilities of Au-phage nanoshuttles to improve the targeted treatment and detection of cancer. Here, we show the application of Au-phage nanoshuttles as the foundation for the development of in vivo and targeted NIR photo-therapies, MRI and CT scanning signal reporting, and NIR surface enhanced Raman scattering (NIR-SERS) detection. As part of this work, we have also begun to assess the toxicity of Au-phage nanoshuttles in the mouse, where no obvious effects associated with nanoshuttle administration were found to affect any of the parameters evaluated in our studies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0775.

P44-8: FLUORESCENT CARBON DOTS AND BIOIMAGING APPLICATIONS

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Clemson University

Fluorescent semiconductor quantum dots have generated much excitement for a wide variety of promising applications, especially those in biology and medicine. For both in vitro and in vivo uses, however, the known toxicity and potential environmental hazard associated with many of these materials may represent serious limitations. Therefore, the search for benign nanomaterials of similar optical properties continues. For quantum-sized silicon, the discovery of Brus and coworkers on the strong luminescence in surface-oxidized nanocrystals has attracted extensive investigations of silicon nanoparticles and nanowires. We have discovered that small carbon nanoparticles (preferably less than 10 nm) can be made highly photoactive upon simple surface passivation, exhibiting strong photoluminescence in both solution and solid-state and with either one- or two-photon excitation. These luminescent carbon nanoparticles ("carbon dots") may find applications alternative to or beyond those of traditional semiconductor quantum dots. For example, they may be derivatized to recognize and bind to biologically active species. Some representative experimental results will be presented, along with discussion on the technology in breast cancer research and imaging applications.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0656.

P44-9: SYNTHESIS AND OPTICAL SPECTROSCOPIC PROPERTIES OF MULTIVALENT AND BIOCOMPATIBLE HYBRID CADMIUM SULFIDE-DENDRIMER NANOCOMPOSITES

Valeria Balogh-Nair, Andrew Byro, Flory Wang, Ronex Muthukattil, Yiu Fat Tse, Hanh Nguyen, Bidyut Baran Das, Robert R. Alfano, and Swapan Kumar Gayen
City University of New York

Background and Objectives: Hybrid nanocomposites of semiconductor quantum dots (QDs) and organic dendrimers are of great interest because their small size, emission tunability, superior photostability and longer luminescence decay times, in comparison with dyes, turn them into promising materials for applications such as contrast agents for biomedical imaging and bioconjugates for nanomedicine. A major challenge is to obtain water soluble biocompatible QDs suitable for the synthesis of bioconjugates designed to prevent breast cancer micrometastases and/or to be used in biomedical imaging application. Here we report on the synthesis of water-soluble nanocomposites of cadmium sulfide (CdS) QDs in polyamidoamine (PAMAM)-amidoethylethanolamine type dendrimer and the characterization of the QDs using absorption and fluorescence spectroscopies.

Brief Description of Methodologies: Cadmium sulfide QDs were synthesized using sixth, fifth, and fourth generation dendrimers (G6-PAMOH, G5-PAMOH, and G4-PAMOH) as nanoreactors. Stock solutions of Cd^{2+} and S^{2-} ion precursors were injected into stirred solutions of the dendrimers in ultrapure degassed water, under argon atmosphere and the mixtures were stirred until maximum growth of the nanoparticles was achieved. Alternatively, QDs prepared in methanol solution could be subsequently transferred into water. The growth of the particles was monitored using ultraviolet-visible absorption and fluorescence spectroscopy. The excitation and emission spectra, as well as their second derivatives, were also measured to establish the emission characteristics of the QDs. The time evolution of fluorescence was measured exciting the samples using the second harmonic radiation from a femtosecond Ti:sapphire laser generating 100-fs pulses at 82 MHz repetition rate. A streak camera measured the time evolution of fluorescence.

Results: All three generations of dendrimers formed fluorescent QDs in methanol solution, with emission maxima at around 470 nm, but only the two larger, fifth, and sixth generation dendrimers afforded nanoparticle synthesis in water. The fluorescence lifetimes measured in water yielded a decay time of 240 ps for the 6GPAMOH-CdS-QDs.

Conclusions: The method reported here is the first straightforward bench top synthesis of water soluble, biocompatible CdS quantum dots at room temperature. Moreover, the dendrimer matrix employed here to stabilize the nanoparticle in water also provides a multivalent platform for interface with biomolecules at the dendrimer's surface. This

interface is critical for the synthesis of QD-bioconjugates designed to prevent breast cancer micrometastases.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0620.

P44-10: RADIOPAQUE, TUMOR-TARGETED NANOPARTICLES FOR IMPROVED MAMMOGRAPHIC DETECTION OF BREAST CANCER

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Background and Objectives: Despite the demonstrated utility of X-ray mammography for breast cancer screening, there is limited ability to image small or noncalcified lesions and to distinguish between benign and malignant microcalcifications. The sensitivity and specificity of mammography could be greatly improved if the X-ray attenuation of breast cancer tissue could be enhanced selectively using tumor-specific antibodies labeled with gold nanoparticles (AuNPs). After i.v. administration, these conjugates would clear rapidly from normal tissue but accumulate at breast tumors. The tumors could then be visualized by X-ray imaging due to the comparatively strong attenuation effect of the gold. Our objective was to perform preliminary studies towards developing gold nanoparticle immunoconjugates and determining their potential use in vivo.

Methods/Results: Initial studies were performed to determine if it would be possible to localize sufficient quantities of gold in tumors to alter the attenuation of X-rays in mammography studies. A 96 well acrylic cell culture plate was employed to represent an array of virtual "tumors." Each cylindrical well was then filled with a prescribed volume and concentration of AuNP suspension, thereby varying both attenuation length and tumor uptake. The entire apparatus was imaged by a conventional film mammography unit. Nanoparticle suspensions were prepared by diluting citrate-stabilized, 10 nm diameter AuNPs (Meliorum Technologies, Rochester, New York) in water. Ten concentrations ranging from 0.01 to 5 wt% gold were prepared in volumes ranging from 50 to 300 μL based on our calculations of expected tumor uptake (data not shown). Various imaging conditions (exposure time, peak kV, etc.) were examined. Quantitative examination by densitometry revealed a detectable increase in attenuation relative to baseline for higher concentrations of nanoparticles. These results suggest that the quantity of gold that must be deposited in a tumor to increase conspicuity should be attainable using conjugates between AuNPs and antibody fragments, particularly if repeat dosing were employed. Attempts to modify gold nanoparticles and render them reactive for conjugation to antibodies using a ligand substitution reaction in the presence of a stabilizing surfactant, e.g., Tween 20 resulted in irreversible aggregation and/or sedimentation of the AuNPs. This resulted in our need to explore alternative strategies. We developed a single-chain Fv (scFv) with C-terminal cysteine residues to facilitate attachment directly to AuNPs using known gold-thiol chemistry. In this scheme, no intermediate step of activating the AuNPs would be required. The gene encoding scFv-cys was successfully cloned and the scFv-cys protein was expressed, purified, and found to be fully functional using methods previously described [*Cancer Res.* 2001, 61:4750]. We are currently attempting to directly conjugate the scFv-cys to the AuNPs.

Conclusions: We have successfully demonstrated that AuNPs can enhance contrast on images obtained on a clinical mammography instrument. We encountered unanticipated difficulties in conjugating the C6.5 diabody to the AuNPs using our proposed methodology and were therefore forced to develop an alternate conjugation strategy, which we continue to evaluate. We are now in position to initiate comprehensive studies that will validate this approach for X-ray mammography contrast enhancement.

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P44-11: TARGETING QUANTUM DOTS TO TUMORS USING ADENOVIRAL VECTORS

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Background/Objective: Despite advances in detection and treatment of cancer, development of novel therapies remains essential in the continuing battle against this disease; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection, or magnetic nanoparticles for magnetic resonance imaging applications. However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the great progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we therefore aim to couple QDs with targeted Ad vectors to achieve specific, selective tumor accumulation. This combination of novel nanotechnology developments and gene therapy targeting strategies is expected to lead to the development of a unique methodology for cancer detection and treatment.

Hypothesis: Ad vectors can be conjugated with QDs, without compromise of vector infectivity, targeting ability, or nanoparticle function.

Experimental Approach: For labeling Ad vectors with quantum dots, a chimeric virus expressing the biotin acceptor peptide in the hexon capsid protein was utilized.¹ This virus is metabolically biotinylated upon replication, facilitating interaction with streptavidin-labeled QDs (655 nm, Invitrogen). The Ad-QD complex was targeted to c-erbB2-expressing breast cancer cells (AU-565) using the previously described bi-functional adapter molecule sCAR-C6.5.² Cells were imaged utilizing Confocal Laser Scanning Microscopy.

Results: Targeted Ad-QDs were taken up by c-erbB2 expressing cells and clearly visible as multiple fluorescent spots in intracellular compartments. In contrast, QDs were not taken up by themselves, nor when coupled to non-targeted Ad vectors.

Discussion/Conclusion: The presented data demonstrates the feasibility of coupling metal nanoparticles such as QDs to targeted Ad vectors. Importantly, Ad retargeting ability upon addition of the nanoparticles remained unaffected. Therefore, Ad can provide a versatile platform for selective binding of nanoparticles, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer through utilization of gene therapy and nanotechnology approaches. This will provide new opportunities for advanced diagnosis and treatment of tumors refractory to the currently available classical therapeutic interventions.

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P44-12: ALTERNATIVE METHODS OF IMAGING MAGNETIC NANOPARTICLES

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Background: Molecular imaging has been the subject of intense and persistent interest because of the potential for understanding both basic biology and pathologic processes. Magnetic particle imaging (MPI) was introduced in 2005 [1] to significant fanfare [2] and is one of the rare methods capable of joining PET, SPECT, and optical in the molecular imaging repertoire. Early estimates of sensitivity are 8 nmol l⁻¹ [3]. MPI is capable of longitudinal studies and is not limited to surface tissues so it promises to be useful for studying metastatic processes.

MPI images are formed by placing nanoparticles in a harmonic magnetic field and measuring the harmonics formed by nonlinearities in the induced magnetization. High sensitivity is achieved because there is no other source of signal at the harmonic frequencies. The original method localized the signal by saturating the nanoparticles everywhere except at a field free point.

We suggest some alternative imaging methods that offer better stability and SNR.

Methods: Simulations were made using a Langevin [4] model for the nanoparticle magnetization as a function of magnetic field bias.

Results: We have previously fit measured signals from iron oxide nanoparticles and iron core nanoparticles to weighted sums of Langevin functions representing log-normal size distributions. The Langevin function is localized in field and has zero mean which are the primary criteria for a wavelet function which can be used to encode images in a variety of ways [5,6,7]. So a linear magnetic field gradient will produce wavelet sensitivity function for signal generation where the size of the applied field gradient determines the scale, or spatial extent, of the basis function and a uniform offset field allows translation.

The simulations show relatively good conditioning for small numbers of encoded voxels over a wide range of gradient fields so a small number of voxels can be encoded using small bias fields. However for large numbers of voxels, the stability suffers significantly. Imaging is still possible but larger gradient fields are required. The best stability is achieved when the distance between the extrema is one voxel that is the minimum-scale wavelet decomposition. The SNR can be increased by adding larger scale basis functions.

Conclusions: MPI is difficult because the size distribution makes saturating the signal outside of the field free point difficult; fields on the order of hundreds of mT will be required. We suggest an alternative method of imaging that activates the second harmonic with a localized field rather than saturating the third harmonic outside the region of interest. We show that the response along a field gradient approximates a wavelet function so it can be used to code images with wavelet-based methods. Relatively large field gradients are required but no larger than those required for the original MPI method. Hybrid methods are also suggested.

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P44-13: NOVEL SIGNAL TRANSDUCTION SYSTEMS FOR NON-INVASIVE BREAST CANCER SCREENING

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The proposed research aims to develop innovative technology that could ultimately lead to new breast cancer screening tests that will be compatible with miniaturized systems integrated in cheap devices suitable for widespread screening of large population groups. It's the ultimate goal of this Idea Award proposal to conduct a proof-of-concept study of the first-time design and evaluation of switchable signal transduction units for screening of breast-cancer indicative biomarkers. In accordance with the guidelines of the Idea Award, this project has been developed as a pilot study to evaluate the feasibility of "smart surfaces" for screening of early-stage breast cancer markers [1-3]. Our specific design hypothesis is that the proper molecular design of these "smart surfaces" will lead to surfaces with dynamically controlled binding events of metabolites indicative of early-stage breast cancer. In contrast to conventional screening approaches, a screening platform that relies on "smart surfaces" for signal transduction will have improved ability to be operated in a complicated environment, such as urine or breath, and thus will be amenable to widespread screening of large population groups. On a basis of a rational modeling approach, we have identified promising initial structures for molecular switches and have worked toward a successful implementation of their synthesis. Self-assembly of monolayers has been conducted with both molecular switches. Monolayers of various molecular switches were assembled on both gold and silver electrodes. When conducting electrochemical impedance spectroscopy under physiological conditions, these monolayers exhibit significant changes in their electrochemical barrier properties upon application of electrical DC potentials below +400 mV with respect to a standard calomel electrode [4]. We further found the impedance switching to be reversible under physiological conditions. Moreover, the impedance can be fine-tuned by changing the magnitude of the applied electrical potential. Before and during impedance switching at pH 7.4 in aqueous buffer solutions, the low-density monolayers showed good stability according to grazing angle infrared spectroscopy data. In an extension of these initial studies, we have examined the storage stability of monolayers made of linear molecular switches for several weeks [5]. Extended stability is an important criterion for selection of suitable signal transduction systems. We further developed technology to microfabricated sensory islands of low-density monolayers in a high-density background of an identical monolayer. These nanostructured surfaces were exposed to elevated temperatures and the effects on film thickness, conformational structure, and electrochemical permeability were studied. Independent of the lateral migration effect, micropatterned SAMs of molecular switches showed thermal stability at temperatures up to 373 K, which is critical for breast cancer diagnosis.

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LIFESTYLE AND NUTRITION II

Poster Session P45

P45-1: ACTIVATION OF STAT5 AND INDUCTION OF A PREGNANCY-LIKE MAMMARY GLAND DIFFERENTIATION BY EICOSAPENTAENOIC AND DOCOSAPENTAENOIC ω -3 FATTY ACIDS

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The protective effect of early pregnancy against breast cancer can be attributed to the transition from undifferentiated cells in the nulliparous to the differentiated mature cells during pregnancy. Considerable evidence suggests strongly that the n-3 polyunsaturated fatty acid (PUFA) content of adipose breast tissue is inversely associated with an increased risk of breast cancer. Here we report that there was a decrease in n-6/n-3 PUFA ratio and a significant increase in concentration of n-3 PUFA DPA and EPA in the pregnant gland. The functional role of n-3 PUFAs on differentiation was supported by the studies in fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Alteration of n-6/n-3 ratio in favor of n-3 PUFA and particularly DPA in the mammary gland of fat-1 mouse resulted in development of lobuloalveolar-like structure and milk protein β -casein expression, mimicking differentiated state of the pregnant gland. DPA and EPA activated Jak2-Stat5 pathway and induced a functional differentiation with production of β -casein. Expression of brain type fatty acid binding protein B-FABP in virgin transgenic mice also resulted in a reduced ratio of n-6/n-3 PUFA, a robust increase in DPA accumulation, and mammary differentiation. These data indicate a role of MRG for preferential accumulation of n-3 DPA and EPA in the differentiated gland during pregnancy. Thus, alteration of n-6/n-3 fatty acid compositional ratio in favor of n-3 PUFA and particularly DPA and EPA is one of the underlying mechanisms of pregnancy-induced mammary differentiation.

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P45-2: EFFECT OF HIGH ω -3 FATTY ACID DIET ON MARKERS OF BREAST CANCER RISK IN POSTMENOPAUSAL WOMEN

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Sex hormone-mediated cancers, such as breast cancer, present a significant problem in the United States. It is important to develop safe and effective preventative strategies for these diseases. Epidemiological evidence and animal studies show that dietary fat is associated with risk of development of sex hormone mediated cancer. Specifically that a high intake of omega (ω)-6 fatty acids increases risk while ω -3 fatty acids are associated with risk reduction. Although the associations between dietary fat and sex hormone mediated cancers is unclear, it is likely due to mechanisms of endocrine balance, eicosanoid production, or immune function.

The primary objective of this investigation is to determine whether diets designed to increase plasma ω -3 fatty acid concentrations (a low fat diet, with or without ω -3 fatty acid enrichment) will favorably affect sex hormone distribution in postmenopausal women in a direction associated with reduced risk of sex hormone-mediated cancer development. The specific aims of this study are to evaluate the effects of total fat and ω -3 fatty acid intake on plasma sex hormone levels in postmenopausal women.

To evaluate these relationships we are conducting a well-controlled feeding study to evaluate dietary fat and fatty acid effects. The diets being tested in 8-week feeding periods include a "high risk" American diet (40% fat; HF), a low fat diet (20% fat; LF), and a LF diet with supplemental ω -3 fatty acids (23% fat; LFn3). Endpoint measures of plasma sex hormones were obtained at baseline and 8 wks of each dietary treatment.

Plasma estradiol (E_2), estrone (E_1), estrone sulfate (E_1 -S), testosterone (T), androstenedione (AS), sex hormone binding globulin (SHBG), follicle stimulating hormone (FSH), dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) were analyzed by radio-immunoassay for 10 participants. SHBG levels were significantly increased at 8 wks with LFn3 compared to LF ($p < 0.05$), and there was a trend for decreased DHEAS level at 8 wks with LFn3 compared to LF ($p < 0.15$). A trend for increased E_2 was observed with HF compared to both LF and LFn3 at 8 wks ($p < 0.15$). A trend for decreased E_1 and FSH was observed from baseline to 8 wks with LFn3 ($p < 0.15$). No statistically significant differences were observed between treatments for A, T, E_1 -S, or DHEA.

Preliminary results suggest that LFn3 alters estrogen metabolism in a direction associated with reducing breast cancer risk in postmenopausal women. LFn3 significantly increased plasma SHBG and decreased DHEAS concentrations in postmenopausal women compared to LF at 8 wks. Within the LFn3 group, trends were observed for decreased E_1 and FSH from baseline to 8 wks. A trend for elevated E_2 level was observed with HF relative to LF and LFn3 at 8 wks. The full effects of the 3 diets on plasma sex hormone profile will be further elucidated as more subjects complete the study.

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P45-3: THE MECHANISTIC ROLE OF IODINE IN BREAST CARCINOGENESIS

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There is both considerable interest and ignorance in the possible role of iodine in the etiology and prognosis of breast cancer. This project is the first step in elucidating a mechanistic role for iodine in breast carcinogenesis. The data that we have been able to generate to date support our hypothesis; namely, using transgenic human breast cancer cells (MCF7) overexpressing the sodium/iodide symporter (NIS) and/or lactoperoxidase (LPO), we have shown that NIS facilitates death or survival pathways following irradiation, a known human breast carcinogen, depending on the presence or absence of iodine, respectively, and that this switching can be modulated by the cell's ability to organify and stabilize the iodine via LPO. Further, we have shown that expression of both NIS and LPO will radiosensitize the MCF7 cells while NIS alone will make them radioresistant and more aggressive. These data agree with observations made by others demonstrating that iodine deficiency is correlated with increased breast cancer incidence and that a large percentage of human breast cancers overexpress NIS. Additionally, the fact that NIS and LPO are most active in the mammary glands during late pregnancy and lactation may explain the well-established observation that parity and lactation history reduce the risk for breast cancer development. We are confident that the data from the experiments currently in progress should help to strengthen our already existing results. Clarification of these issues should foster future studies not only in breast cancer diagnosis and therapy but also in prevention through conscious changes in diet and environment.

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P45-4: ALTERED MUTATION LOAD IN ADIPOSE TISSUE IN BIG BLUE MICE WITH CHRONIC CONSUMPTION OF DIETARY ANIMAL FATS

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Breast cancer is the most common female cancer and a major cause of morbidity and mortality, with more than 200,000 new cases and 40,000 deaths in the United States in 2005. Despite intensive study, the origins of sporadic breast cancer are largely unknown. Genetic susceptibility, hormonal effects and environmental factors can increase the incidence of breast cancer. Epidemiological studies suggest that environmental factors may contribute to 80% of breast cancer in the US. Herein we test the predictions of **lipophilic mutagen hypothesis of breast cancer** (LMHOB): mammary epithelial cells and adipocytes are preferential targets of mutagenesis by lipophilic mutagens transferred through the food chain from plants to animal fat to human adipose tissue. Adipocytes concentrate lipophilic mutagens and mammary cells are the cancer prone cells preferentially affected given the unique anatomy of mammary tissue. An alternate hypothesis to LMHOB is that fat consumption causes mutagenic oxidative stress in mammary cells. As a pilot test, mice were fed chemically defined diets containing a high percentage of animal fat (35% each of beef or pork fat) beginning at birth. The fats came from California, Louisiana or Iowa. Plant oil (25% soybean oil) and standard 9% fat diets were controls. Mutation load was assessed in adipose tissue (reservoir of lipophilic mutagens), colon epithelium (cancer prone epithelia), and cerebellum (90% neurons) in Big Blue[®] mice fed high fat diets for eight to ten months. Upon screening 32.7million plaque-forming units, we collected 1680 mutants, sequenced 1557 mutants (1.72 megabases) and found 1195 independent (non-clonal) mutations. Certain predictions of LMHOB were confirmed: i) the mutation pattern in adipocytes in mice fed 35% beef fat diets from California is significantly different from those in mice fed standard Purina diet ($p = 0.002$); ii) mutation patterns of adipocytes in mice fed beef fat diets from Iowa and California differed dramatically ($p = 0.009$), resulting from region-specific deviations from the control diet, and iii) high fat diets did not alter the patterns of mutations in colonic epithelium or neurons. The results are most consistent with regional differences in lipophilic mutagens dissolved in fat, rather than any endogenous mutagenic action of fat. Tests of additional predictions of LMHOB are in progress. Currently, mice treated with the high fat diets for twenty months are being analyzed.

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P45-5: FOLATE AND DNA METHYLATION IN A MOUSE MODEL OF BREAST CANCER

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Preclinical animal models are invaluable resources for assessing the influence of dietary and epigenetic factors on tumorigenesis. However, investigation of the effects of dietary folate and DNA methylation on mammary tumorigenesis in animal models has

been surprisingly limited. A particularly good animal model of human breast cancer is the polyomavirus (PyV) middle T (mT) transgenic mouse. These mice initially develop normal mammary ductal trees, but then progressively develop premalignant mammary intraepithelial neoplasias (MIN, equivalent to ductal carcinoma in situ or DCIS in humans), invasive carcinomas, and ultimately pulmonary metastases. The mT antigen overexpressed in this model mimics the gene product erbB2 (aka HER-2), which has been implicated as a key molecule in human breast cancer. In preliminary studies, we have demonstrated that a methyl-deficient diet (deficient in folate and choline, and low in methionine) slows the growth of MIN and malignant transformation. We have also demonstrated by immunohistochemistry that epigenetic factors, including DNA methyltransferases and methyl-CpG-binding proteins are highly expressed in normal ductal cells, MIN, and resulting tumors, suggesting a role of these proteins in the tumorigenesis process.

An important refinement of the PyV-mT mouse model has been the development of transplantable MIN outgrowths (MIN-Os). MIN-Os, which are maintained by serial transplantation into cleared fat pads of nontransgenic mice, have reproducible biological endpoints, including tumor latency and metastasis rates. MIN-Os are therefore a relevant preclinical model of human DCIS and tumorigenic transformation that are amenable to investigation of chemopreventive and dietary interventions.

Our research objective is to assess the influence of dietary folate and demethylation of DNA on the transformation of premalignant mammary lesions to malignancy using the MIN-O model. MIN-O lines with previously established tumor latency and incidence rates will be transplanted into cleared mammary fat pads of mice fed folate-replete (control), folate-deficient, or folate-excess diets. Additional mice receiving MIN-O transplants will be fed the control diet along with chronic administration of the DNA-demethylating agent, 5-aza-deoxycytidine (ADC). MicroPET imaging will be used to monitor MIN-O growth and tumorigenic transformation in vivo. Tumor latency and incidence, pathological characterizations, gene expression profiles, gene-specific promoter methylation, and gene targeting by methyl-CpG-binding proteins will be compared among the treatment groups.

It is expected that these studies will demonstrate roles of folate and DNA methylation in mammary tumorigenesis and will identify specific hypermethylated genes and associated methyl-CpG-binding proteins that contribute to the transition from premalignant mammary lesions to malignancy. Importantly, these studies will not only focus on folate deficiency but will also assess the effect of excess dietary folic acid on malignant transformation, an issue of concern since the fortification of cereal and grain products with folic acid in the late 1990s.

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P45-6: ADIPOSE STEM CELLS AS POTENTIAL PROMOTERS OF BREAST CANCER PROGRESSION

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Introduction/Objective: Obesity, manifested by overgrowth of white adipose tissue (WAT), a widespread problem among middle-aged women, has been associated with advanced progression of breast cancer. The mechanism of obesity influence on cancer is not understood. We hypothesized that adipose tissue directly promotes cancer through its effect on formation of new blood vessels, the process necessary for expansion of tumor mass. While the involvement of circulating endothelial progenitors in tumor vasculogenesis and their cancer-stimulatory effect has been demonstrated, the origin of these cells has remained controversial.

Methods: We proposed to test a novel idea, according to which tumor vasculogenesis can be promoted by adult adipose stem cells (ASC), which are abundant in white fat. It is possible that ASC of obese patients are recruited as a source of vascular precursors for tumor blood vessels. Because ASC have been shown to secrete growth factors inducing cell proliferation, ASC could also indirectly promote cancer through stimulating proliferation of endothelial and tumor cells. To explore the role of adipose-derived stem cells in promoting breast cancer, we used the mouse tumor model for experiments that test whether ASC can home to tumors and promote tumor growth.

Results and Conclusions: We show that adipose-derived stromal cells home to breast tumors in the mouse model, engraft into tumor vasculature, and functionally engage into vasculogenesis. Our results have important implications for our understanding of obesity as a cancer risk factor and for reconsiderations of approaches to breast cancer management. This study also calls attention to adult stem cells, projected for therapeutic applications, as a potential complicating factor in cancer patients.

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P45-7: PREGNANCY WEIGHT GAIN, HORMONE AND GROWTH FACTOR LEVELS, AND BIOMARKERS OF BREAST CANCER RISK IN NIPPLE ASPIRATE FLUID

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Pregnancy is a period when the mammary gland undergoes extensive cell proliferation and differentiation in response to gestational hormones and growth factors. Excessive weight gain during pregnancy is a risk factor for breast cancer. To characterize the biological changes linked to excessive weight gain during pregnancy, we investigated the association between body weight and circulating hormone levels in pregnant women. Further, we sought to examine the impact of pregnancy weight gain and hormone levels on breast cancer risk-related characteristics (color and volume) of nipple aspirate fluid (NAF) samples collected 1-year postpartum. Pregnant women (n=373), ages 25 to 45, were recruited at the Maternity Clinic at Solna, Stockholm, Sweden between 1997 and 2004. All participants signed an informed consent form and visited the clinic on pregnancy weeks 12, 22, and 32. A 3-day 24-hour dietary record, pregnancy body weight, and a blood sample were obtained for each subject during these visits. One year after delivery, subjects returned to clinic and body weight and blood and NAF samples were obtained from the study participant. The protocol was approved by the institutional review boards at Georgetown University and at the Maternity Clinic at Solna, Sweden. Estradiol (E2) and progesterone (P) levels were measured using RIA assays. Levels of leptin and insulin like growth factor 1 (IGF-1) were determined using an EIA assays. IGFBP-3 levels were determined by the Immulite assay. Sera Coloration of breast fluids was determined, using a visual scale, as colorless, white, pale yellow, dark yellow, brown, green, or black. The volume of NAF collected from each subject was measured by recording the length of the column of fluid inside each capillary tube in millimeters (1 mm in the capillary tube = 1 μ L of fluid). Blood samples were obtained from 283 women and NAF samples from 129 women. Leptin levels increased during pregnancy and peaked on the second trimester. Levels of E2, P, IGF-1 and IGFBP-3 increased as pregnancy advanced and reached the highest levels on week 32 of pregnancy. Levels of E2 and P sharply decreased 1 year after the end of pregnancy. IGF-1 and IGFBP-3 levels were also reduced 1 year after delivery, but the decrease was modest, especially for IGFBP-3. The volume of NAF ranged from 0.5-143.4 μ L, with a mean of 44.9 ± 38.0 μ L. Most NAF samples (69%) were categorized as pale-yellow. The remaining samples were distributed among colorless, white and dark yellow categories. Comparison of pregnancy leptin and IGF-1 levels at the three pregnancy time-points and 1-year post-partum with characteristics of NAF samples indicated that NAF volume is positively associated with IGF-1 ($p=0.027$) and leptin ($p=0.031$) levels in the first and second trimesters of pregnancy, respectively. These preliminary results suggest that pregnancy leptin and IGF-1 levels are linked to NAF-related biomarkers of increased breast cancer risk. The completion of pregnancy hormone assays and characterization of the remaining NAF samples and their cellular content will allow us to determine whether any of these parameters correlate with excessive pregnancy weight gain. Future studies include identification of dietary factors linked to weight gain during pregnancy, pregnancy hormone and growth factor levels and biomarkers of breast cancer risk in NAF, enabling us to develop novel intervention strategies to prevent breast cancer.

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P45-8: INHIBITION OF MYOSTATIN FAILS TO INHIBIT MUSCLE WASTING IN CANCER CACHEXIA

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Background/Objective: Over a third of breast cancer patients suffer from cachexia, defined as debilitating progressive loss of adipose tissue and skeletal muscle not due to starvation. Cachexia itself is responsible for 25%–33% of cancer deaths. Myostatin is a member of the Transforming Growth Factor- β superfamily expressed in skeletal muscle, which inhibits muscle hypertrophy and hyperplasia. Mice, dogs, and humans deficient in myostatin show a 2–5-fold increase in skeletal muscle mass. Conversely, we have shown that myostatin overexpression causes cachexia. Myostatin can be inhibited by follistatin, which can be induced with trichostatin A (TSA), a histone deacetylase inhibitor. TSA has been shown to reduce muscle degeneration in a model of muscular dystrophy. We sought to determine whether inhibiting myostatin pharmacologically or genetically might preserve muscle in cancer cachexia.

Methods: In the genetic model, C57BL/6J myostatin null (*Mstn*^{-/-}) mice and age (~12 wk) and sex-matched wild-type controls were injected with B16.F10 melanoma cells, a well-characterized model of cancer cachexia. Mice were weighed and tumors measured daily and tissues dissected at euthanasia. In the pharmacological model, CD2F1 mice

were injected with colon-26 (C26) adenocarcinoma cells. Once tumors were visible (at 9 days after injection), TSA was administered by daily i.p. injection (0.6 mg/kg body weight in DMSO) for 7d prior to euthanasia. Control C26 mice received DMSO only. Non-tumor-bearing mice received either TSA or DMSO on the same schedule as tumor-bearing mice. Follistatin and myostatin mRNA levels were assayed by quantitative real-time RT-PCR.

Results: Tumor size, fractional weight loss, and fractional muscle mass were not different in *Mstn*^{-/-} versus wild-type mice (Student's t-test). However, there was a trend toward greater fat loss (35% ± 20.2 versus 20.35% ± 19.45%, n = 12 per group, p = 0.0844), along with increased markers of inflammation, including greater total spleen mass (p = 0.01) and greater liver to body mass ratios (p < 0.0001) at lower fractional tumor burdens in *Mstn*^{-/-} mice. This result indicates that *Mstn*^{-/-} mice are not protected from cancer cachexia and moreover may actually be more sensitive to tumor-induced inflammation. In the pharmacological model, TSA treatment induced follistatin expression 2.5-fold in non-tumor-bearing mice and 1.5-fold in C26 mice. Moreover, TSA reduced myostatin expression 50% in non-tumor-bearing mice and 20% in C26 mice. Consistent with reduced myostatin activity, the gastrocnemius from TSA only mice were 22% larger than DMSO only controls (p < 0.001). TSA treatment did not protect mice from C26 wasting, however. Percent body weight loss, fat loss, and skeletal muscle loss were statistically indistinguishable in TSA- versus DMSO-treated C26 mice.

Conclusion: Neither genetic nor pharmacological inhibition of myostatin activity was sufficient to prevent or reduce muscle wasting in two models of cancer cachexia. These results suggest that despite their promise in muscle dystrophies, myostatin inhibitors may not be effective in diseases associated with chronic inflammation.

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P45-9: RATIONALE AND DESIGN OF A RANDOMIZED, CONTROLLED TRIAL INVESTIGATING THE EFFECTS OF AEROBIC EXERCISE ON TUMOR VASCULARITY AND RESPONSE TO NEOADJUVANT THERAPY IN OPERABLE BREAST CANCER PATIENTS

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Rationale: Neoadjuvant or adjuvant chemotherapy for women with locally advanced breast carcinoma of the breast is now accepted as an effective treatment modality to improve relative risk of recurrence and death by up to 30%. Unfortunately, most solid tumors are resistant to chemotherapy as a result of poorly developed vascular systems. One pleiotropic intervention that may favorably modulate the tumor vasculature is aerobic exercise training. However, no study to date has explored the effect of exercise training on tumor vascularization and response in women undergoing chemotherapy for operable breast cancer.

Study Design: The trial is divided into two distinct phases: In Phase 1, using a single-group design, 3 participants are identified and screened for eligibility via medical record review of patients scheduled to initiate anthracycline-cyclophosphamide (AC) neoadjuvant chemotherapy at Duke. Following the successful completion of all baseline assessments, all patients receive chemotherapy plus aerobic exercise training. In the absence of these effects, using a two-armed, prospective, randomized design, 20 patients are randomly assigned to chemotherapy alone or chemotherapy plus aerobic exercise training. Patients assigned to aerobic exercise training perform an individualized exercise prescription consisting of three cycle ergometry sessions per week at approximately 60%–80% of baseline exercise capacity on nonconsecutive days for the duration of neoadjuvant AC chemotherapy (approximately 12 weeks).

Objectives: The primary aim of Phase 1 is to establish whether combining exercise training with chemotherapy leads to unacceptable exercise adherence rates or unusually high dose-limiting toxicities (DLTs). In the absence of these effects, the aims of Phase 2 are to determine the effects of aerobic exercise plus chemotherapy versus chemotherapy alone on (1) tumor physiology (blood flow, microvessel density), (2) systemic response (circulating angiogenic factors, endothelial function, and exercise capacity) and (3) tumor response (pathologic and clinical responses). A final specific aim will be to determine and define patient eligibility and recruitment as well as identify a primary end point to calculate sample size for a future adequately powered Phase 3 trial.

Hypotheses: In Phase 1, we hypothesize that patients receiving chemotherapy plus aerobic exercise training will achieve acceptable exercise adherence rates (>70% of total number of planned sessions) and normal dose-limiting toxicities (DLTs). In Phase 2, we hypothesize that patients assigned to chemotherapy plus aerobic exercise training will have (1) significantly higher tumor blood flow and lower microvessel density (MVD); (2) serial increases in circulating levels of angiogenic factors, higher endothelial function, and exercise capacity; and (3) higher pathologic and clinical tumor response than patients assigned to chemotherapy alone.

Preliminary Results: Phase 1 has been successfully completed with acceptable adherence and no unusual DLTs. To date, 7 patients have been randomized in Phase 2 (chemotherapy plus aerobic exercise, n=4; chemotherapy alone, n=3). Five patients have completed the study and the overall adherence rate is 88% (range: 75%–100%). We anticipate completing the trial in January 2009 with a final sample of 20.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0336.

P45-10: ROLES OF IRON DEFICIENCY AND OVERLOAD IN BREAST CANCER OUTCOMES OF PRE- AND POST-MENOPAUSAL WOMEN

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Pre-menopausal breast cancer (BC) patients are known to have a higher risk of dying from the disease than older patients because of greater early recurrence rates and aggressiveness of their tumors. Specific risk factors contributing to this high BC recurrence in pre-menopausal patients have not been identified. On the other hand, BC incidence rate is higher in post- than in pre-menopausal women. Yet, serum circulating estrogen levels are lower in post- than in pre-menopausal women. How a low level of estrogen contributes to a high BC incidence in post-menopausal women is not completely understood. In the present study, we have tested a hypothesis that in pre-menopausal women, an iron deficiency due to menstruation, along with estrogen, stabilizes hypoxia inducible factor-1 α and increases vascular endothelial growth factor (VEGF) formation. This mechanism makes the pre-menopausal patients more susceptible to angiogenesis and, consequently, leads to higher recurrence rates. Conversely, increased iron levels in post-menopausal women due to menstrual period cessation contribute to higher BC incidence rates via oxidative stress pathway. Because animal models simultaneously mimicking menopausal transition of both iron and estrogen levels are lacking, we first tested our hypothesis in a tissue culture model of high estrogen and low iron (17 β -estradiol and apo-transferrin) or low estrogen and high iron (holo-transferrin and ferritin), simulating pre- and post-menopausal conditions. We found that BC MCF-7 cells and primary human normal epidermal keratinocytes (NHEK) cells grown under pre-menopausal conditions lead to significantly higher levels of VEGF than those from the same cells grown under post-menopausal conditions. By testing each component separately, we further found that estrogen slightly up-regulated VEGF and it was ferritin that significantly suppressed VEGF. Using human biopsies from pre- and post-menopausal skin, it was found that levels of ferritin were higher but VEGF lower in post- than in pre-menopausal skin and, interestingly, levels of ferritin inversely correlated with levels of VEGF. These in vivo data support our observation in an in vitro menopausal system. On the contrary, more alterations in oxidative stress were observed in cells grown under post- as compared to the same cells grown under pre-menopausal conditions. Using Affymetrix genechip[®] technology, approximately 140 genes were shown to be altered in primary NHEK cells by post-menopausal conditions. There were no gene modifications in cells grown under pre-menopausal conditions as compared to cells without treatment. Our study indicates that iron, a growth nutrient as important as estrogen in female metabolism and development, and its change before, during, and after menopausal transition, may hold key information and explanations to BC outcomes.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0717 and National Cancer Institute (R21 CA132684).

P45-11: INHIBITION OF MAMMARY TUMORIGENESIS BY DIETARY PHYTOCHEMICALS

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Breast cancer is a leading cause of cancer-related deaths among American women. The American Cancer Society estimated 242,540 new cases and 40,910 deaths due to breast cancer in 2007. The etiology of majority of breast cancers is multifactorial, of which 95% is environmental and hormonal. Polycyclic aromatic hydrocarbons (PAHs) are commonly found in our environment and appear to be carcinogenic by genotoxic mechanisms. The detection and characterization of aromatic DNA adducts related to PAHs exposure in breast cancer patients suggest an important role of DNA adducts in breast cancer.

Many molecular and cellular studies have revealed the ability of dietary phytochemicals in vegetables and fruits to inhibit mammary tumors. The major mechanisms of chemoprevention involve induction of phase II detoxification enzymes and/or inhibition of phase I enzymes that are involved in the activation of certain carcinogens. The abundant and inexpensive chemopreventive dietary phytochemicals are also being explored as phospho-glycoprotein (P-gp) modulators for the reversal of multi drug resistance (MDR) to breast cancer chemotherapeutic drugs.

Studies in our laboratory revealed that in female mice treated with 7,12-dimethylbenz-(a)anthracene (DMBA), the mammary EROD activities, CYP1A1, 1B1, and hepatic AHR mRNA expressions were increased significantly when compared to controls. Significant reduction of these gene expressions along with elevation of several phase II enzymes were observed in female mice treated with chemopreventive phytochemicals, sulforaphane and curcumin. Another group of mice were fed with sulforaphane and curcumin after exposure to DMBA. The hepatic and mammary EROD activities and CYP1A1/1B1 mRNA levels (real-time RT-PCR) were determined. DMBA-mediated induction of hepatic and mammary CYP1A1/1B1 expression was suppressed significantly by chemopreventive compounds. However, the chemopreventive compounds were more effective when fed to mice before exposure to DMBA.

The mammary P-gp expression was elevated in mice treated with chemotherapeutic drugs, paclitaxel and docetaxel, compared to controls. Administration of dietary chemopreventive phytochemicals, sulforaphane and curcumin, inhibited the P-gp expression significantly showing a potential to reverse MDR.

These studies with naturally occurring dietary constituents will provide information that will help eliminate breast cancer in women. These studies will also help to develop rational strategies for the combination drug development for the successful chemotherapy of environmental- and hormonal-induced human breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0296.

P45-12: OBESITY AND PERINATAL TCDD EXPOSURE INCREASES MAMMARY TUMOR INCIDENCE IN FVB MICE

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Background and Objectives: Breast cancer risk consistently correlates with total lifetime exposure to estrogens. Obesity, which creates more estrogen-producing adipocytes and stores more lipophilic toxins, increases breast cancer risk. Because both lipophilic 2,3,7,8-tetrachloro-p-dioxin (TCDD) and adipocytes impact the estrogen pathway, we examined how TCDD and obesity interact to alter mammary cancer susceptibility.

Methodologies: At 12.5 days post conception, we exposed FVB females to 1 µg/kg of TCDD or vehicle. To model diet-induced obesity, the nursing dams were put on high or low fat diets at birth. Female offspring were kept on the same diets after weaning exposed to DMBA at post-natal days (PND) 35, 49, and 53 and monitored for tumor development. A second FVB cohort was treated identically up until PND 50, when mammary gland mRNA was analyzed by microarrays and real-time PCR.

Results: TCDD and HFD appeared to synergistically increase mammary tumor prevalence. ERα mRNA was higher in mammary tumors than in mammary tissue. Maternal TCDD exposure increased ERα mRNA in both mammary tissue and tumors compared to vehicle. High fat diet increased mammary gland metabolism and genes involved in cancer.

Conclusion, Including Potential Impact: In summary, obesity increases the sensitivity of FVB to DMBA-induced mammary carcinogenesis. Perinatal TCDD exposure

interacts with HFD to increase mammary cancer incidence, possibly through upregulation of ERα. Should this result be replicated in human studies, it would support a need for risk assessments to evaluate the obese for their unique susceptibility to TCDD exposures.

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P45-13: MODIFICATION OF BRCA1 BREAST CANCER RISK BY COFFEE

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The purpose of this study was to investigate the role of coffee and caffeine in the function of the DNA repair protein BRCA1 and to determine whether coffee and/or caffeine prevent BRCA1 hereditary breast cancer. We have bred the necessary genetically engineered mice for the animal study to determine whether coffee, decaffeinated coffee, or caffeine prevents BRCA1 hereditary breast cancer. We applied for a no-cost extension to complete these studies during the next year so we should know whether caffeine prevents breast cancer in the animal model. We have also analyzed the 1853 mutation in the BRCA1 mutant cell line HCC1937 and shown that it is unaffected by DNA damaging agents including caffeine although the full length wild-type BRCA1 protein is affected. We also show that the BRCA1 mutant proteins 1853 and Cys61Gly show a loss of nuclear localization and are defective in DNA repair and radiation response.

To study the biochemical properties of mutant BRCA1 in comparison to wild-type protein, we utilized recombinant human adenoviruses expressing BRCT truncated (Ad-1853), RING mutated (Ad-C61G), or wild-type (Ad-BRCA1) protein. These adenoviruses contain an R-G-D modification in the fiber knob surface binding protein, which increases the efficiency of infection and downstream expression of the transgene. Transduction of the BRCA1 mutant HCC-1937 human breast carcinoma cell line with these viruses at a multiplicity of infection (MOI) of 100 viral plaque forming units per cell yielded approximately equal protein expression of all three forms of BRCA1 48 hours following viral transduction. Analysis of control cells infected with Ad-GFP showed that almost no endogenous BRCA1 protein can be detected in this cell line. Expression of BARD1 and BACH1, which is another BRCA1-interacting protein involved in DNA repair, was found to be approximately equal in all transduction groups (data not shown). Prior to harvest, quantitation of GFP expression in Ad-GFP transduced cell cultures by microscopy indicated that the transduction efficiency is approximately 80% for this cell line under these conditions. We determined the subcellular localization of these forms of BRCA1 by immunofluorescence microscopy. A dual anti-BRCA1 antibody protocol was utilized to ensure the specificity of the staining protocol. C61G BRCA1 was organized into discrete nuclear foci, similar to the expected focal nuclear pattern observed with the wild-type protein. However, 1853 stop BRCA1 was primarily localized in the cytoplasm. Similar to immunoblot results, we found that virtually no endogenous BRCA1 could be detected by this staining protocol.

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EPIDEMIOLOGY II

Poster Session P46

P46-1: IS BI-ILIAC BREADTH A PREDICTOR OF EARLY MENARCHE?

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Few, if any, of the established risk factors for breast cancer are modifiable late in life. Some critical ones such as early menarche and a woman's reproductive hormone profile, are determined early in life, a time when there may be opportunities for primary prevention. The growth of pubertal girls is characterized by broadening of their pelvis with an increase in the width between the iliac crests and rounding of the crests in anticipation of childbirth. Menarche has been linked to fat mass especially that of the trunk. We have been conducting a prospective study in adolescent girls to better understand those breast cancer risk factors linked to early maturation. We report here on the determinants of early menarche. A group of white, Asian, and mixed white-Asian girls identified through a health maintenance organization on Oahu, Hawaii, were first examined at mean age 10.9 years (range: 9.1–13.9 years) and examined twice more, at mean intervals of 2.1 ± 0.1 and 3.3 ± 0.3 years, respectively, for dietary intake (3-day food records), skeletal lengths and breadths, adiposity, sexual maturation (Tanner staging) and bone mass using standardized methods. Indices of central and peripheral adiposity were constructed by summing skinfolds measured on the trunk (subscapular and iliac skinfolds) and on the limbs (biceps, triceps, and calf skinfolds). Among the 87 girls prepubertal at the first exam who completed follow-up, all but 16 reached menarche by the third exam. Cox proportional hazard regression was used to identify predictors of menarche during follow-up. Adjusting for age, height at baseline, and percent Asian ethnicity, shorter time to menarche was found to be strongly associated with bi-iliac (maximum pelvic) breadth ($p < 0.001$). Further adjustment for trunk skinfolds and peripheral skinfolds strengthened this association. A strong correlation (Spearman $r = 0.73$) existed for bi-iliac breadth at the 1st and 3rd exam, suggesting that this measure may track over time. In a cross-sectional analysis of the cohort examined at the 1st exam ($n = 198$), bi-iliac breadth was independently associated positively with trunk-to-periphery fat ratio measured by skinfolds ($p < 0.003$), height ($p < 0.001$), Tanner pubic hair development ($p < 0.001$) and calories from protein ($p < 0.001$), and negatively with calcium intake ($p = 0.01$). These relationships were weaker in a similar analysis at the second exam ($n = 106$), except for the strong association of bi-iliac breadth with trunk-to-periphery fat ratio measured by DXA or skinfolds (both $p < 0.001$). These data suggest that bi-iliac breadth might be an early and persistent marker of early maturation. If this is confirmed, bi-iliac breadth could be used as an intermediate endpoint in intervention studies aimed at preventing early maturation and as a marker of breast cancer risk would remain to be investigated.

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P46-2: ARE OLDER PERSONS DIFFERENT? RESULTS OF THE BREAST CANCER INTERNET INFORMATION AND SUPPORT (BCIIS) STUDY

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Abstract: The Breast Cancer Internet Information and Support study (BCIIS) is an online needs assessment that asks persons with breast cancer what types of features they would most like to see in Internet-based programming related to breast cancer. Preliminary analyses indicate significant differences in characteristics of older respondents related to physical and mental health as well as online support needs.

Introduction: Breast cancer is a significant problem in our society. As informational and support options on the Internet proliferate, it becomes important to differentiate what types of programming are needed and desired by different subsets of persons who have breast cancer.

Older persons are increasingly using the Internet and bring different sets of needs than their younger counterparts. The aim of this project is to present differences in older persons with breast cancer related to a number of variables. The Breast Cancer Internet Information and Support (BCIIS) Survey began in 2004 and is ongoing. Preliminary analyses indicate that important differences exist between older and younger persons with breast cancer.

The Breast Cancer Internet Information and Support (BCIIS) Survey: Subjects were recruited from hospitals and clinics, oncologists' offices, and on web sites and electronic mailing lists related to breast cancer. Instruments used were demographic and medical history surveys and the attitudes toward online healthcare (ATOHC) and SF-36 surveys. The survey was completed entirely online at the web site <http://breastcancersurvey.med.yale.edu>.

Results: Preliminary analyses of $n = 417$ respondents indicate that older persons take significantly more time to complete the survey. As expected, they tend to be retired or working part-time. They are primarily caucasian and live in the United States. They

have more energy difficulties in attending land-based groups and more physical difficulties but feel mentally better than their younger counterparts.

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P46-3: EPITHELIAL CELLS IN NIPPLE ASPIRATE FLUID AND SUBSEQUENT BREAST CANCER RISK: A HISTORIC PROSPECTIVE STUDY

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Background: Past studies have shown that women with abnormal cytology or epithelial cells in nipple aspirate fluid (NAF) have an increased relative risk (RR) of breast cancer when compared to women from whom NAF was attempted but not obtained (non-yielders). This study analyzed NAF results from a group of women seen in a breast clinic between 1970-1991 ($N = 2480$). Our analysis presented here is an aggregate of two sub-groups: women with questionnaire data ($n = 712$) and those with NAF visits beginning in 1988 ($n = 238$), the year in which cancer case information was uniformly collected in California.

Methods: Cytological classification was determined for a group of 946 women using the most abnormal epithelial cytology observed in fluid specimens. Breast cancer incidence and mortality status was determined through June 2006 using data from the California Cancer Registry, California Vital Statistics and self-report. We estimated odds ratios (ORs) for breast cancer using logistic regression analysis, adjusting for age. We analyzed breast cancer risk related to abnormality of NAF cytology using non-yielders as the referent group and breast cancer risk related to the presence or absence of epithelial cells in NAF, using non-yielders/fluid without epithelial cells as the referent group.

Results: Overall, 10% (93) of the 946 women developed breast cancer during the follow-up period. Age-adjusted ORs and 95% confidence intervals (C.I.) compared to non-yielders were 1.4 (0.3 to 6.4), 1.7 (0.9 to 3.5), and 2.0 (1.1 to 3.6) for women with fluid without epithelial cells, normal epithelial cells, and hyperplasia/atypia, respectively. Comparing the presence or absence of epithelial cells in NAF, women with epithelial cells present in NAF were more likely to develop breast cancer than non-yielders or women with fluid without epithelial cells ($RR = 1.8, 1.1$ to 3.0).

Conclusions: These results support previous findings that (1) women with abnormal epithelial cells in NAF have an increased risk of breast cancer when compared to non-yielders or women with normal epithelial cells in NAF, and (2) women with epithelial cells present in NAF have an increased risk of breast cancer when compared to non-yielders or women who had NAF without epithelial cells present.

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P46-4: A NOVEL APPROACH TO DETECT THERAPEUTIC RESISTANCE IN BREAST CANCER

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Introduction: Globally, increasing breast cancer incidence rates, improved prognosis, and growing life expectancy have resulted in increasing number of women at risk of developing a bilateral primary breast cancer. Hence, optimal surveillance and clinical management of women who have had one or two primary breast cancers is a challenge. Our aim is to understand whether adjuvant therapy of a first primary breast cancer might predict the prognosis of a metachronous bilateral cancer.

Materials: Our cohort for analysis of possible therapeutic resistance in short latency bilateral cancer comprised 17,089 women; among these women, 441 developed metachronous bilateral disease within 5 years of their primary breast cancer during follow-up through 1999. We included only women with TNM stage 1–3 at first diagnosis to minimize the risk of misclassified metastatic disease and further to minimize confounding by indication for adjuvant treatment in relation to bilateral breast cancer death. For all these women, all medical records were retrieved and information on treatment, tumor characteristics, hormonal information, etc., was ascertained and computerized. To date, 91% of 441 women with metachronous cancers diagnosed within 5 years have been retrieved, and these women were included in the analysis.

Methods: The occurrence of distant metastasis was used as a measure of prognosis. Incidence rate of distant metastasis within 5 years after the diagnosis was calculated with the accumulated person-time at risk as the denominator. This time started at second diagnosis for bilateral breast cancer and continued until occurrence of distant metastasis, emigration, death, or end of follow-up (December 31, 1999), whichever came first. Poisson regression was used for modeling of occurrence of distant metastasis. Follow-up was censored at age 80 years because classification of cause of death may be less reliable in older women.

Results: Women who had received any form of systemic adjuvant treatment were 3.2 times (95% CI 1.1–9.0) more likely to develop a distant metastasis compared to women who received no adjuvant treatment when we adjusted for follow-up time, age, calendar period, TNM stage, hormone receptor status, and adjuvant treatment of the second cancer. Women who received adjuvant chemotherapy were 4.7 times (95% CI 1.4–16.1) more likely to develop a distant metastasis compared to women who received no adjuvant treatment with similar adjustment. Since the collection and preparation of tissue samples is still ongoing, we did not start with analysis of tissue samples. Therefore, our focus was so far on the setup and standardization of QM-FISH (quantitative multigene fluorescence in situ hybridization), a method that will be used to study copy number changes (allelic imbalances) in about 50 selected genes. As a result of methodological development in Anders Zetterberg's lab, several individual genes can now be identified and quantified accurately and reproducibly in each tumor cell nucleus at the same time, which makes the technique suitable for large-scale clinical studies.

Conclusions: Women with bilateral breast cancer have a poor survival. Predictors of a poor outcome include young age at first cancer, a second diagnosis within 5 years, and those treated with adjuvant therapy for the first cancer.

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P46-5: ABSTRACT WITHDRAWN

P46-6: RISK FACTORS FOR DEVELOPING BREAST CANCER FOLLOWING BENIGN BREAST DISEASE: A 25-YEAR FOLLOW-UP OF A NATIONWIDE COHORT

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Background and Objectives: Benign breast disease (BBD) increases risk for later breast cancer (BC) by about 50%, with more proliferate and atypical categories conferring higher risk. Between June 1979 and June 1980, our group undertook a nationwide study to identify all (2,448) new cases of BBD in Israel; histopathology material was independently re-examined. In 1994, a 15-year follow-up of the BBD cohort for morbidity from BC was performed; 1,756 women were located and interviewed to evaluate risk factors for BC. The small number of BCs that had occurred till then (109) limited the analysis. The objectives of the current study were:

1. Estimate the association between BBD and BC risk by histopathological type after a 25-year follow-up.
2. Assess to what extent other known risk factors for BC modify the risk for developing BC, in women with previous BBD.
3. Evaluate the possible stepwise progression hypothesis of BBD (from nonhyperplastic to hyperplastic to atypia to DCIS and invasive cancer).

Methodology: BC and vital status were updated through 2005 by linkage to the Israel National Cancer and Population registries. Histological classifications were made according to the criteria of Page and grouped into 4 categories (non-proliferative changes, proliferative changes without atypia, proliferative changes with atypia and others). Standardized incidence ratios (SIRs) were computed as a ratio of observed to expected cases of BC, with 95% confidence intervals (CI), for the total group and in strata. Odds ratios (ORs) were calculated to evaluate risk factors for BC using logistic regression. The stepwise progression theory was evaluated by tracking histology categories of subsequent BBDs and by comparing rates of ipsilateral and contralateral BCs.

Results: By the end of 2005, 185 BC cases had developed yielding an increased risk of 62% (95% CI 1.4–1.87) among the BBD cohort compared to the general Israeli population (controlling for age and ethnic origin). While the SIR for non-proliferate lesions was not significant, the SIRs for proliferate lesions with atypia reached 3.32 (1.81–5.57) and without atypia 1.79 (1.49–2.14). This risk was found for women who were pre- and peri-menopause at the time of their BBD but not for women who were older than 55. The excess risk remained for the entire follow-up period.

For developing BC, a protective effect for more births, young age at first delivery and long periods of breast feeding were found. However, no modification effect between BBD and any of the known risk factors of BC was found. A non-significant elevated risk of 1.22 (0.91–1.65) was seen for developing an ipsilateral BC after BBD.

Conclusions: Our results agree with other publications showing a positive association between degree of proliferation and atypia of the BBD lesion and risk for BC. Persistence of risk seen for 25 years following the BBD diagnosis and the fact that the majority of women at risk are diagnosed before the recommended screening age raises the need for developing screening and other prevention policies in women with BBD. Individual risk must take into account a previous BBD as well as other known risk factors. These results necessitate patient as well as physician education regarding the long-lasting future risk of BBD.

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P46-7: SCREENING MAMMOGRAPHY IN OLDER WOMEN: EFFECT OF WEALTH AND PROGNOSIS

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Background: Wealthy women have higher rates of screening mammography than poor women. Screening mammography is beneficial for women with substantial life expectancies, but women with limited life expectancies are unlikely to benefit. It is unknown whether higher screening rates in wealthy women are due to increased screening in women with substantial life expectancies, limited life expectancies, or both. This study examines the relationship between wealth and screening mammography use in older women according to life expectancy.

Methods: Cohort study of 4,222 women, 65 years or older with Medicare participating in the 2002 and 2004 Health and Retirement Survey. Women were categorized according to wealth and life expectancy (based on 5-year prognosis from a validated prognostic index). The outcome was self-reported receipt of screening mammography within 2 years.

Results: Overall, within 2 years, 68% of women received a screening mammogram. Screening was associated with wealth (net worth >\$100,000) and good prognosis (<10% probability of dying in 5 years). Screening mammography was more common among wealthy women than among poor women (net worth <\$10,000) both for women with good prognoses (82% versus 68%, $p < .001$) and for women with limited prognoses ($\geq 50\%$ probability of dying in 5 years), (48% versus 32%, $p = 0.02$). These associations remained after multivariate analysis accounting for age, race, education, proxy report, and rural residence.

Conclusion: Poorer older women with favorable prognoses are at risk for not receiving screening mammography when they are likely to benefit. Wealthier older women with limited prognoses are often screened when they are unlikely to benefit.

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P46-8: ABSTRACT WITHDRAWN

P46-9: RISK OF HYPOTHYROIDISM IN OLDER BREAST CANCER PATIENTS TREATED WITH RADIATION

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Background: Hypothyroidism is a potential complication after radiation therapy (RT) when treatment fields include the thyroid. We assessed risk of hypothyroidism in breast cancer patients receiving RT to a supraclavicular field, which typically includes a portion of thyroid.

Methods: We identified 38,255 women (age >65) without history of hypothyroidism from the Surveillance Epidemiology and End Results (SEER)-Medicare cohort diagnosed with stage 0-III breast cancer from 1992–2002 and 111,944 cancer-free controls. We compared hypothyroidism incidence among irradiated patients with 4+ positive lymph nodes (4+LN, surrogate for supraclavicular RT) and no positive nodes (0 LN, surrogate for no supraclavicular RT); nonirradiated patients; and controls. Proportional hazards models tested associations of LN, RT, and breast cancer status with hypothyroidism.

Results: The 5-year incidence of hypothyroidism was identical (14%) in irradiated patients with 4+LN, 0 LN, and nonirradiated patients ($p = 0.52$). After adjusting for sociodemographic and clinical characteristics, hypothyroidism risk was not increased in irradiated patients with 4+LN versus 0 LN (hazard ratio [HR] = 1.04, 95% Confidence Interval [CI] 0.89, 1.23). However, all patients, irrespective of RT status, were more likely to be diagnosed with hypothyroidism compared with cancer-free controls (HR = 1.21, 95% CI 1.17, 1.25).

Conclusion: Development of hypothyroidism is fairly common in older breast cancer survivors. Though supraclavicular irradiation does not appear to amplify risks, further studies on the role of routine thyroid function monitoring in all breast cancer patients regardless of treatment status may be warranted given the excess risks compared with the general population.

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P46-10: BREAST DENSITY ASSESSMENT IN PUBERTAL GIRLS USING DXA: A FEASIBILITY STUDY

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Rationale: Breast density is a strong predictor of breast cancer risk that can be obtained from digitized mammographic images of women at recommended screening ages. This study explored the use of a dual energy x-ray absorptiometer (DXA) device to measure breast density in pubertal girls with various degrees of breast development.

Methods: We recruited 18 girls (13–14 years) who indicated their current breast development using a line drawing depicting the five Tanner breast stages (Figure 1). Two dedicated DXA scans of their left and one of their right breasts were taken on a GE Lunar Prodigy Bone Densitometer. The scans were calibrated to a 2-compartment model of fat (steric acid) and dense fibroglandular tissue using phantoms of different thickness and density. Total breast area was independently and manually delineated on each image and % fibroglandular volume (%FGV), absolute fibroglandular volume (FGV in cc), and total breast area and volume (TV) were computed.

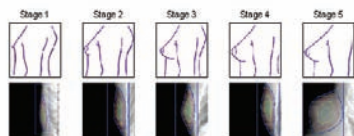


Figure 1. Breast developmental stages according to Tanner with the respective DXA images

Results: It was possible to image all five Tanner stages. The DXA showed the breast tissue separate from the pectoral muscle and rib cage. The %FGV ranged from 31.9 to 92.2% (mean = $71.1 \pm 14.8\%$) while FGV ranged from 80 to 270 cc (mean = 168 ± 54 cc). Left and right whole breast %FGV densities were highly correlated ($r = 0.97$, $p < 0.0001$) and of the same magnitude ($p = 0.18$). However, FGV and TV of the left breasts were larger than the right by 19 cc ($p = 0.02$) and 38 cc ($p = 0.04$), respectively. FGV and TV increased by Tanner stage whereas %FGV density did not. BMI was significantly related with %FGV ($r = -0.68$, $p = 0.003$) and TV ($r = 0.56$, $p = 0.02$) but not with FGV ($p = 0.95$). The precision of the method was excellent for %FGV (RMS SD = 2.4%) and moderate for FGV (RMS SD = 16.6 cc).

Conclusion: DXA breast scans may be a novel technique to image the developing breast in studies examining breast cancer risk in early life. We will conduct a study among 100 women and their pubertal daughters with the following specific aims:

1. Correlate DXA breast density measures with mammographic density among adult women
2. Compare the association of known breast cancer risk factors with DXA breast density to their association with mammographic density among adult women
3. Describe DXA breast density by Tanner stage of breast maturation among pubertal girls
4. Relate DXA breast density to other observable measures of pubertal maturation
5. Examine the relation between breast density measured by DXA in mothers and daughters

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0489 and National Center for Research Resources (P20 RR11091).

P46-11: DISPARITIES IN STAGE AT DIAGNOSIS IN ORANGE COUNTY: IMPLICATIONS FOR EARLY DETECTION

Sarah Frances Marshall,¹ Hoda Anton-Culver,¹ Diana Chingos,² Cheryl Cooky,³ Travers Ichinose,⁴ Sandra Rose,⁵ Deborah Ryan,⁶ Raul Sobero,⁴ Lisa M. Wolter,⁶ Argvrios Ziogas,¹ and Chrisina Tannous⁶

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This is a collaborative data analysis project conducted by researchers from the University of California, Irvine under the guidance of the Orange County Affiliate of Susan G. Komen for the Cure, and the project's Community Advisory Group. In Orange County, cancer data is routinely collected by the California Cancer Registry; the project aim is to analyze existing data in order to inform decisions of local service provision.

In the first of a series of analyses, the goal was to discover who was more likely to be diagnosed with breast cancer at a late stage. An early-stage diagnosis is usually detected by mammographic screening so by identifying the groups likely to have a late-stage diagnosis, we can locate who would benefit from improved screening services. This is particularly important because stage at diagnosis considerably affects prognosis. We hypothesized that there were racial/ethnic and socioeconomic disparities in stage at diagnosis in our local community.

Description: The Advisory Group consists of representatives from the local health department and from community-based organizations that fund or provide breast cancer services; more than half of the members are breast cancer survivors. The group is responsible for selecting the topic and for leading and overseeing the project. We publish results in publicly available monographs; the Advisory Group and scientists work closely on the contents and wording of these to ensure that all scientific terms are adequately defined and that the findings can be fully understood by community workers and the general public.

For the analyses, logistic regression is used to calculate the probability of late-stage disease among breast cancer diagnoses and to look for characteristics that may be associated with late-stage diagnosis.

Summary of Results: We found that women younger than 50 years are less likely to be diagnosed with breast cancer but for those who are, their chances of being diagnosed at a late stage are much higher than for older women. The proportion of late-stage diagnoses among breast cancers differed significantly according to religion, marital status, health insurance, neighborhood socioeconomic status, and residence. A likelihood of a late-stage diagnosis differed by race/ethnicity with the chance of being diagnosed late higher for Pacific Islanders and African Americans than for non-Hispanic Whites even after multivariable adjustment. In multivariate models, the most significant characteristics associated with a late-stage diagnosis were younger age, lower socioeconomic status, and having no health insurance.

Conclusions: There are racial/ethnic, socioeconomic, and age disparities in stage at diagnosis locally. Community organizations can use this information to target the provision of breast cancer screening services toward the groups of most need; being able to identify women at greatest risk for diagnoses with the worst prognoses is essential for utilizing limited resources. The program is ongoing with the publication of our first monograph expected in early 2008, followed by three more in the same year, as well as community presentations of the results.

This work was supported by Susan G. Komen for the Cure.

BEHAVIORAL SCIENCES

Poster Session P47

P47-1: ISOLATION AND THE TIMING OF MAMMARY GLAND DEVELOPMENT, GONADARCHE, AND OVARIAN SENESCENCE: IMPLICATIONS FOR MAMMARY TUMOR BURDEN

Martha McClintock
University of Chicago

In this study of Norway rats, we hypothesized that lifelong psychosocial experiences, social isolation, or group living trigger different developmental trajectories in the ovarian system, contributing to pre-disease pathways for spontaneous mammary tumors. Epidemiological studies indicate that early puberty and delayed menopause are risk factors for breast cancer. To that end, we took a cross-sectional prospective approach and examined the ovarian system at two developmental points, young adulthood and middle age. We assessed ovarian function at both points, as well as mammary gland development at puberty and mammary tumor burden in middle age. Social isolation dissociated two components of puberty; it accelerated maturation of ovarian function while it simultaneously delayed mammary tissue development thereby increasing the exposure of developing breast parenchyma to high levels of estrogen. By mid-life, socially isolated rats had greater mammary tumor burden despite having entered estropause prematurely, demonstrating that isolation did not increase tumorigenesis by prolonging ovarian function. These findings are discussed in the context of facultative lifespan strategies for coping with stressors while living in isolation or in a large community.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0296.

P47-2: TESTS OF A PREDICTIVE MODEL OF ADOLESCENT FUNCTIONING TO MATERNAL BREAST CANCER

Frances Lewis and Kristin A. Fletcher
University of Washington

Background and Objectives: In 2007, an estimated 178,480 women in the United States were diagnosed with invasive breast cancer; an additional 62,030 had in situ disease. Approximately one-fourth of these women had an adolescent child, yielding 52,912–60,127 adolescents. Despite the large numbers affected, there has been limited research on adolescents' adjustment to maternal breast cancer, and interventions have lagged far behind actual need. The aim of this study was to test a theoretical predictive model of adolescent functioning to recently diagnosed nonmetastatic maternal breast cancer.

Methods: The current study involved secondary analysis of baseline data from 222 study participants in 2-parent families with an adolescent. Mothers (67%) were diagnosed 6 or fewer months and most (78.4%) were on active treatment. Children averaged 15.7 (SD 1.8) years, 58% of whom were male.

Results: The reduced model for mothers' data showed that more pressures from the cancer on the diagnosed mother increased her depressed mood ($Beta = .57, p < .001$), and this negative mood tended to negatively impact adolescent competence ($Beta = -.020, p = .055$). Higher maternal parenting quality predicted higher self-esteem ($Beta = -.47, p < .001$) as well as higher adolescent functioning (Externalizing CBCL $Beta = .31, p < .05$, Total CBCL $Beta = -.26, p < .05$). The reduced model for fathers' data showed that greater pressures from the illness on fathers increased their depressed mood ($Beta = .68, p < .001$), and this negative mood predicted lower marital adjustment ($Beta = -.43, p < .001$). The father's negative view of his marital adjustment significantly predicted lower adolescent competence ($Beta = .26, p = .01$). Better parenting quality by the father predicted higher self-esteem (Rosenberg, $Beta = -.52, p < .001$). Finally, parenting quality and adolescent competence predicted separate aspects of the adolescents' functioning. The Rosenberg Self-Esteem scale predicted fewer Internalizing Problems ($Beta = .29, p < .05$), and fathers' parenting quality predicted fewer Externalizing Problems ($Beta = -.26, p = .05$). All zero order paths were tested and were nonsignificant.

Conclusion: Based on study results, a multicomponent intervention is being developed for pilot testing.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0577 and University of Washington.

P47-3: NF- κ B AS A CRITICAL BIOLOGICAL LINK BETWEEN PSYCHOLOGICAL STRESS AND BREAST CANCER

Fiona Yull,¹ Linda Connelly,¹ Leshana Saint-Jean,¹ Taylor Sherrill,¹ April Newsome,¹ Rachel Pigg,¹ and Barbara Fingleton²

¹Vanderbilt University Medical Center and ²Vanderbilt-Ingram Cancer Center

It is a widespread belief that psychological stress is a major factor in breast cancer. However, the biological pathways that link stress to increased breast cancer risk are not well understood. The nuclear factor-kappaB (NF- κ B) family of transcription factors is recognized as linking inflammation and immunity to cancer. NF- κ B signaling is positioned as a pivotal regulator of aberrant responses that lead to cancer. We have tested the hypothesis that NF- κ B is a critical biological link between psychological stress and breast cancer. We used reporter transgenic mice that express a luciferase/GFP fusion

protein in response to NF- κ B activity to measure NF- κ B responses to acute and chronic stress. To model acute stress we restrained mice for short periods of time. To model chronic stress we housed animals in either 1/cage (solitary) or 10/cage (overcrowded) conditions as opposed to 5/cage (control). We measured the NF- κ B response in mammary and lung tissue by luciferase assay. We then combined the established PyVT model of breast cancer with our reporter mice in models of chronic stress to determine effects on NF- κ B signaling and on breast cancer progression. We monitored both primary mammary tumor development and subsequent metastasis to the lungs. Our data suggest that NF- κ B activity can be increased in response to both acute and chronic stress and that this impacts both primary tumor formation and subsequent metastasis to the lung. As stress is such a prevalent factor in our lives, a better understanding of how this may impact breast cancer risk should be a high priority.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0575.

P47-4: BREAST CANCER SURVIVORSHIP: PSYCHOSOCIAL CHALLENGES AND HEALTH BEHAVIOR OF FIRST DEGREE FEMALE CAREGIVERS

Victoria H. Raveis, Sheindy Pretter, and Monique Carrero
Columbia University School of Public Health

Background and Objectives: Cancer survivorship is now broadly defined to include those who have been impacted by cancer, such as first-degree relatives of breast cancer patients who are at increased risk of developing breast cancer. Women are a primary source of emotional support and informal aid to ill relatives. Through their careprovision to a breast cancer patient women gain intimate knowledge of the cancer experience. This investigation examined the breast cancer experience and the psychosocial issues confronting first-degree female relatives caring for a breast cancer patient. The study objective was to obtain information about the attitudes, beliefs, and health practices of female caregivers at increased risk for breast cancer due to family history and to describe the psychological stresses and challenges they experience.

Methodology: A diverse sample of 100 adult first-degree female relatives caring for their mother, sister, or daughter diagnosed with breast cancer completed an in-depth interview. Surveys were collected from the patients. Data was collected in English or Spanish. Interviews were audiotaped, transcribed, and subjected to thematic analysis. For the caregiver-patient dyad to be eligible, the patients had to be in active outpatient treatment for breast cancer. The caregiver had to be 18 or older; a first-degree female relative (i.e., a daughter, sister, or mother); not have a personal history of cancer; and have been involved in the provision of practical assistance and/or emotional support to the patient since her breast cancer diagnosis.

Findings: Analyses documented that the diagnosis of cancer was an event of significant import to family functioning. Female caregiving relatives experienced a period of crisis fraught with severe emotional distress and life/death concerns that parallels the existential plight patients encounter. The breast cancer diagnosis was an event that made female caregiving relatives realize their vulnerability. Caregiving relatives struggled with ambivalence and uncertainty regarding personal risk while being called upon to provide support. The intimate knowledge caregiving provided of the breast cancer experience impacted caregivers' sense of risk, increased illness-related concerns, informed attitudes and beliefs about the disease, and affected their health behavior. Caregivers reported elevated levels of perceived risk. They also worried how this risk may impact future generations. For some, genetic testing helped resolve the ambiguity about their risk. The caregiving experience translated into some women being more proactive about their health. For others, this experience was associated with intrusive thoughts, worry and anxiety, and hypervigilance.

Conclusions: Cancer control efforts that promote adherence to screening guidelines for at-risk women are key to the early detection of breast cancer. The study contributes to the limited understanding of the cancer experience on first-degree relatives and the impact of this experience on their health promotion activities. Such insights are critical to developing outreach programs and interventions tailored to address the concerns and needs of at-risk women and enable them to make informed choices in managing their risk and optimizing their health.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0215 and New York State Department of Health.

P47-5: CLOSE RELATIONSHIP QUALITY IN BREAST CANCER PATIENTS: PREPARING TO TEST A BIOMARKER

Karen Louise Weihs
University of Arizona, Tucson

Background and Objectives: Weihs documented improved survival in women with more close relationships.

Specific Aims for the Project: (1) Test the association between the quality of intimate relationships and plasma oxytocin.

Methods: Study Design: Assess relationship quality and oxytocin every 3 months for 2 years.

Measures of Relationship Quality: Dyadic Adjustment Scale (DAS); Multidimensional Scale of Perceived Social Support (MSPSS); Social Relationships Inventory (SRI) Measures of Individual Characteristics promoting high quality interpersonal relationships: Adult Attachment Scale(RAAS); Mini-K Scale (Factor K)

Results: Number of subjects completing each of 8 total visits: Visit 1 = 98; Visit 2 = 81; Visit 3 = 64; Visit 4 = 49; Visit 5 = 22; Visit 6 = 12, Visit 7 = 4; Visit 8 = 1. Oxytocin levels: Plasma stored but not yet analyzed. The relationship quality measures are performing as expected, with higher correlation between DAS and SRI ($r=.48$), than between DAS and MSPSS($r=.22$). The DAS is the only relationship quality indicator correlated with individual characteristic measures: RAAS($r=.28$) and Factor K (.27).

Conclusions: (1) This 5-year study has been enrolling subjects for 2 years and is on track to complete enrollment of 168 subjects by 9/2008. (2) Assessments of relationship quality and individual characteristics promoting close relationships will provide strong indicators of these constructs for correlation with oxytocin levels when these are analyzed.

Weihis KL, *Psychosomatic Medicine* 2008; 70 (1):117-124

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0603.

P47-6: BREAST CANCER RISK FACTORS AND IMMUNE RESPONSES IN HEALTHY PREMENOPAUSAL WOMEN

Na-Jin Park and Duck-Hee Kang
University of Alabama at Birmingham

Breast cancer is a complex disorder, development of which is influenced by multiple risk factors. Family history of breast cancer, especially in first-degree relatives (FDRs), has been identified as an important risk factor and used in assessing an individual risk of breast cancer development. Furthermore, women with a FDR with breast cancer showed lower immune responses, such as natural killer cell activity (NKCA) and interferon-gamma (IFN- γ), than those without any family history. Using multiple risk factors, breast cancer risk assessment models, including the Gail model, have provided a better tool of predicting objective breast cancer risk. However, it is difficult to ensure clinical significance of the model, partly because its associations with breast cancer-relevant biological mechanisms, such as immune response, have rarely been tested. Examining the associations of other potential risk factors (e.g., current birth control use in premenopausal women, psychological distress) with immune responses would be a way to confirm its clinical significance as a risk factor of breast cancer. Both NKCA and IFN- γ are one of important early tumor defense mechanisms.

The specific aims of this study were: (1) to examine the association of objective breast cancer risk assessed by the Gail model with immune responses (NKCA and IFN- γ); (2) to determine an individual contribution of each risk factor included in the Gail model to immune responses; and (3) to examine the association of other potential risk factors of breast cancer, such as current birth control use and psychological distress, with immune responses in healthy premenopausal women.

For this cross-sectional study, a convenience sample of 93 healthy premenopausal women (mean age = 32.2) completed questionnaires and gave a blood sample for immune measurement. A chromium-51 release cytotoxicity assay determined NKCA, whereas enzyme-linked immunosorbent assay (ELISA) determined IFN- γ production.

Regression analyses revealed that higher objective breast cancer risk was associated with lower NKCA at 12.5:1 effector-to-target ratio (NKCA 12.5) ($p=.044$) and IFN- γ ($p=.012$) in healthy premenopausal women. The combination of 5 risk factors showed significant associations with NKCA 12.5 ($p=.001$), but not with IFN- γ ($p=.107$). Decreased NKCA 12.5 was associated with late first live birth or no birth, increased number of FDRs with breast cancer, and being African American in this study sample of women. Current age and age at menarche were not associated with any immune parameters. In addition, current birth control users showed lower NKCA at all four effector-to target ratios than no users. There were no associations between psychological distress and immune responses.

Although some significant relationships were found between objective risk and immune responses, individual contributions of each risk factor to an immune parameter seem to be different. Current birth control use showed a consistent relationship with decreased NKCA, which can be significantly considered in predicting objective risk of premenopausal women. The findings of this study may provide details useful in developing breast cancer prevention strategies such as early identification of at-risk individuals before its clinical expression. Further investigation is warranted in this area.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0352.

P47-7: EFFECT OF LIGHT PERCEPTION ON REPRODUCTIVE FUNCTION IN BLIND WOMEN

Erin E. Evans,¹ Richard G. Stevens,² and Steven Lockley¹

¹Brigham and Women's Hospital and ²University of Connecticut Health Center

The role of the circadian system in human reproductive development is unclear. Women with varying degrees of blindness, which is known to affect circadian rhythmicity, are reported to have altered reproductive function and a lower risk of breast cancer compared to sighted women. These differences have been attributed in part to photoreceptor dysfunction in the blind. Currently, the causes of these differences in reproductive function remain unknown. The present study was conducted to determine whether differences exist among reproductive measures in legally blind women (Snellen Scale 20/200 or less) with at least light perception (LP) as compared to those with no perception of light (NPL), and how these differences may relate to breast cancer risk.

Survey data were collected from 1,358 legally blind women (LP, $n = 958$; NPL, $n = 400$; age range 19-98 yrs) across the United States and Canada in the format of their choice. General and reproductive characteristics were compared between LP and NPL subjects to determine whether visual acuity was related to reproductive development and reproductive outcomes. Statistical analysis was conducted using Student's two-sample t tests to compare demographic variables of interest. Odds ratios and 95% confidence intervals were estimated using multivariate logistic regression.

We found that NPL women reported having an earlier menarche than LP women (12.16 ± 1.53 and 12.45 ± 1.57 yrs, respectively; $p<0.01$). This effect was enhanced when women who reported having NPL from birth were compared to all other participants (11.94 ± 1.67 and 12.4 ± 1.55 yrs; $p<0.01$). Early menarche was significantly associated with being NPL in unadjusted (OR: 0.88 [0.82-0.96], $p<0.01$) and adjusted multivariate analyses (OR: 0.91 [0.84-0.99], $p<0.05$, adjusted for Body Mass Index, current age, height). We also found that NPL women were less likely to report any full-term pregnancy compared to LP women (OR: 0.54 [0.42-0.68], $p<0.01$); however, NPL women who reported at least one full-term pregnancy were more likely to report a history of breastfeeding than LP women (OR: 1.63 [1.12-2.36], $p<0.01$). Start and end of natural menopause, age of first-term pregnancy and hormone replacement therapy use were not significantly different among LP and NPL blind women.

Our findings have important implications on how light may disrupt the circadian and reproductive systems although how these effects may relate to cancer risk is unclear. Further research exploring the interaction between light, reproductive development and breast cancer are warranted.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0553 and Harvard Medical School (HL07901-08).

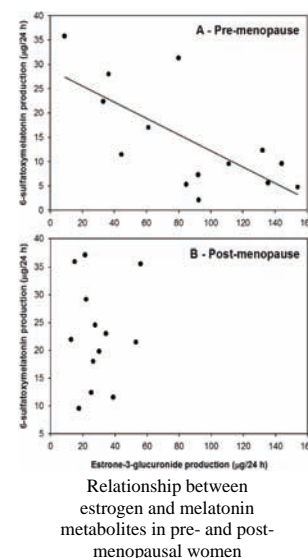
P47-8: URINARY MELATONIN AND ESTROGEN PRODUCTION IN PRE- AND POST-MENOPAUSAL BLIND WOMEN

Steven Lockley and Erin E. Evans
Brigham and Women's Hospital

Epidemiological observations indicate that the risk of breast cancer is lower in people who are visually impaired compared to the sighted population and that risk is inversely correlated with visual acuity. Melatonin has been shown to have oncostatic properties in animal models of breast cancer. The "melatonin hypothesis" suggests that blind people are less susceptible to suppression of melatonin by exposure to light at night and may therefore have higher melatonin levels. Frequent light-induced melatonin

suppression has been hypothesized as a cause of the higher breast cancer incidence observed in female night shift workers and flight attendants. The aim of this study is to investigate further the relationship between severity of blindness and melatonin production while also assessing how melatonin and estrogen may be related.

We studied 130 visually impaired women during an 8-week field study. Subjects were asked to complete daily sleep logs and collect sequential 4-8-hourly urine samples for 48 h on 2-3 occasions for assessment of melatonin and estrogen metabolite production. Preliminary analysis of the first 27 subjects (14 premenopause, mean age \pm SD = 40.1 ± 7.9 yrs; 13 post-menopause, 55.8 ± 4.3 yrs) showed a significant difference in the relationship between melatonin and estrogen production between pre- and post-menopausal women. Pre-menopausal subjects produced significantly more urinary estrone-3-glucuronide/24 h than



post-menopausal subjects, as expected (pre-; $86.4 \pm 45.5 \mu\text{g}/24 \text{ h}$ versus post; $29.2 \pm 13.4 \mu\text{g}/24 \text{ h}$; $p < 0.001$, two-tailed t-test). They also produced significantly less 6-sulfatoxymelatonin/24 h than post-menopausal women (pre-; $14.5 \pm 10.8 \mu\text{g}/24 \text{ h}$ versus post; $23.1 \pm 9.3 \mu\text{g}/24 \text{ h}$; $p < 0.001$). Furthermore, in the pre-menopausal women alone, a significant negative relationship was observed between the melatonin and estrogen metabolite output/24 h ($R^2 = 0.495$, $p < 0.01$, Figure A). A similar relationship was not observed in the post-menopausal subjects, primarily due to the low estrone levels (Figure B). These data provide encouraging preliminary evidence that melatonin and estrogen are reciprocally associated and that the reduction in estrogen production associated with menopause may result in higher melatonin production. Our future work will examine how this association relates to breast cancer risk and light exposure.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0553 and Harvard Medical School (HL07901-08).

P47-9: SPATIALLY INFORMED INVESTIGATIONS OF RACE-SPECIFIC SOCIAL GRADIENTS IN BREAST CANCER DISPARITIES

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Background: The purpose of this disparities research is to examine the extent to which social class, health behaviors, and access to health care, measured at the community level, explain geographic variation in breast cancer burden among African American and white women in the state of Maryland. The goal of this exploratory scientific activity is to elucidate possible proximal mechanisms that drive enduring but poorly understood associations between African American ethnicity and aggressive breast cancer biology, late-stage diagnosis, and excess disease burden. Although ethnic disparities are often speculated to be caused by social and economic resource differentials, few studies have examined the race-specific role of social resources on breast cancer outcomes or explicitly tested how specific elements of social class actually drive biological outcomes. Thus, it is difficult to identify which aspects of social class disparities should be prioritized for more refined studies or for cancer control planning and interventions.

Methods: All cases of breast cancer reported to the Maryland Cancer Registry from 1992 through 2003 are being used for this research. All cases are being geocoded by residential location within the state, and using all available clinical information from the registry record, multiple breast cancer outcomes are being explored. Variation in incidence, histologic grade and other tumor characteristics, stage of disease at diagnosis, and to the extent data permit, estrogen receptor status, lymph node involvement, and survival, are being examined, using multilevel modeling. Outcomes will be modeled as a function of individual case characteristics, including age and year of diagnosis, as well as Census area behavioral profiles, socioeconomic resources, and availability of health services. Analyses will identify single behaviors as well as composite characteristics of communities having excess breast cancer burden for African American cases and compare these significant characteristics to those associated with excess burden for white cases. Finally, spatial analysis techniques, including unadjusted and adjusted cluster analyses, will examine the extent to which these significant behavioral and social risk factors explain geographic patterns of breast cancer disparities in the state.

Results and Conclusions: To date, project activities have involved the negotiation of data agreements between the Maryland Cancer Registry and the researchers and the identification of case selection criteria and variables of interest. A total of 95 variables will be received on more than 51,000 cases of breast cancer reported during this 12-year window. Methodologic findings will include discussion of the creation of case data files for analysis, including geocoding, creation of disease characteristic measures from registry data, and creation of behavioral, social, and health resource measures from existing area-level data. In addition, preliminary findings, including frequencies and bivariate associations, will be discussed. Finally, the applicability of the research methodology developed for this disease-specific example will be considered for inquiries into other disease areas where disparities may be rooted in part to social or behavioral risk factors.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0451 and Maryland Cancer Registry of the Maryland Department of Health and Mental Hygiene.

P47-10: A RANDOMIZED TRIAL OF COMPUTER- AND PAPER-BASED VERSIONS OF THE DECISION BOARD FOR BREAST CANCER TREATMENTS

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¹McMaster University and ²Juravinski Cancer Centre

Background: Women with breast cancer want to be involved in treatment decision making. We have developed and evaluated the Decision Board (DB), which is a visual aid administered by the clinician during the consultation. Randomized trials have dem-

onstrated that the DB (called the standard version) improves patients' comprehension, satisfaction, and comfort with decision making. Despite these benefits the DB is not widely used in part due to the lack of access and perceived difficulty in use. Computer-based instruments are attractive as they provide more versatility in presenting information and can be easily updated and accessed via the Internet. A potential concern is that they may be awkward to use in the consultation. We developed computer and paper-based versions of the DB for primary surgical therapy (mastectomy or breast conserving therapy) and adjuvant chemotherapy (no treatment, CMF, AC or AC+T). The objective of this study was to compare the computer- and paper-based versions of the DB to the proven standard version for patient knowledge, decisional conflict, and satisfaction with decision making.

Methods: A total of 309 women were randomized to the physician consultation plus either the standard (105), computer- (103) or paper-based (101) versions of the DB. Patients were stratified according to whether they were making a decision regarding surgical treatment or adjuvant chemotherapy. Patients were evaluated following the consultation for their knowledge about the breast cancer treatments offered, decisional conflict, and satisfaction with preparation for decision making using established instruments. Usefulness of the instrument for the patient and physician was also assessed.

Results: Patient knowledge, decisional conflict, and satisfaction with preparation for decision making were similar between the three versions of the DB (Table). Usefulness of the instrument for the patient and physician was similar for the different versions.

Outcome (scale)	Standard (mean)	Computer (mean)	Paper (mean)	p-value
Knowledge (1-100)	77.9	78.3	78.7	0.94
Decisional Conflict (1-5)	1.79	1.85	1.79	0.80
Satisfaction (0-4)	3.12	3.03	3.15	0.57

Conclusion: No differences were observed between the computer- and paper-based versions of the DB in comparison to the standard version suggesting that either version may be used to support patient involvement in decision making. Given the proven benefits of the DB, computer- or paper-based version may facilitate wider under use of this patient-based decision aid.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8100.

P47-11: A WEB-BASED EDUTAINMENT DECISION AID FOR LOW HEALTH LITERACY WOMEN MAKING BREAST CANCER TREATMENT DECISIONS

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¹Baylor College of Medicine, ²University of Texas Health Science Center at Houston,

³Texas Cancer Center, and ⁴Baylor College of Medicine Breast Center

Providing decision support to patients with low health literacy is a challenging and under-researched area. With funding from the BCRP (DAMD17-2-98-8022), we developed and evaluated a computerized patient decision aid to assist women with early-stage breast cancer in making a surgical treatment decision. The decision aid incorporates entertainment-education (edutainment) with enhanced (factual) content. This project was designed to educate low health literate women and novice computer users. An interactive jewelry box is featured to assist women in recording and reflecting over issues of concern with possible treatments, deliberating over surgery decision, and, communicating with physicians and significant others. Findings from the study indicated that women who received usual care plus the patient decision aid made more informed treatment decisions than women who only received usual care. Women who viewed the patient decision aid found the application easy to use and understand, informative, and enjoyable and were less worried about treatment.



Screen shot of updated, web-based patient decision aid

Implementation of the aid on a broader scale has been limited by a number of factors: (1) the content was outdated, (2) the application did not run in newer computer systems, and (3) the application could run only from a hard drive and was not design for the Internet. Recently, the Susan G. Komen for the Cure provided us with funding to update, package, and disseminate the software to national audiences.

To accomplish the objectives, the reference document of the existing decision aid was reformatted and improved. Then, the reference document was reviewed and updated by a content expert panel. Concurrent with the review of the content, usability testing of the existing decision aid was conducted with novice computer users to evaluate and improve the usability of the program. Based on findings from the usability testing, significant modifications were made to the user interface and functionality. In addition,

the architecture of the decision aid was converted to an open-architecture that permits efficient content management, deployment, and updating.

Currently, we are in the final phase of programming. Once completed, we will conduct additional usability testing of the updated application with novice computer users. Lastly, the updated decision aid will be installed in a dedicated public web site hosted by the Dan L. Duncan Cancer Center, a NCI-designated cancer center. Availability of the patient decision aid will be announced at the Era of Hope 2008 meeting. A brief demonstration of the decision aid will be provided.

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P47-12: EXPLORING WOMEN'S DECISION-MAKING EXPERIENCES ABOUT BREAST CANCER TREATMENT THROUGH VIDEO-STIMULATED RECALL INTERVIEWS

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Background: Women with breast cancer (BC) desire information, in part, to be involved in treatment decision making (TDM). However, several researchers have reported that patients' actual experiences in TDM have not matched their preferences. This study's objectives were to identify processes of TDM used by women with BC and to identify physicians' behaviors that facilitated or impeded women's involvement in TDM.

Methods: A qualitative approach with video-stimulated recall interviews was used. Surgical (n=6) or medical oncology consultations (n=15) with new BC patients were videotaped. Subsequently, women and surgeons or medical oncologists separately viewed their consultation while being interviewed. Interviews were taped, transcribed, and analyzed.

Results: Most women described an iterative TDM process where they obtained information about treatment options from social networks and identified preferred treatment options prior to the consultation. All women reached an agreement about the type of surgery with their surgeon during the consultation. At the post-surgery appointment, most women wanted their surgeons to give them more detailed information about tumor pathology and potential treatments offered by medical oncologists to help them prepare for subsequent TDM. Most women deliberated about adjuvant systemic therapy options both during and after the medical oncology consultation and reached a decision several days post-consultation. Surgeons and oncologists described many behaviors that they used to facilitate women's involvement in TDM. While women identified many of the same behaviors as the physicians reported, they also described different behaviors. Women identified more items related to patient-physician rapport than did physicians. Women also identified that physicians helped to involve them in TDM when they explicitly explained the rationale for patient involvement in TDM, used visual aids to explain treatment options, offered a treatment recommendation that provided reassurance, and indicated that women had time to make treatment decisions. Physicians identified more specific information-giving behaviors than did women. Women identified relatively few physician barriers to their involvement in TDM. The most frequently mentioned barrier related to lack of preparation for chemotherapy discussions.

Conclusions: Many women with BC identified several TDM processes including information gathering, identification of preferred options, deliberation about treatment options, and reaching agreement with their physician on the type of treatment to be implemented. Most women perceived that TDM involved several processes that occurred over time. These findings have implications for researchers who are interested in measuring patient involvement in TDM. Family physicians and surgeons are important in the TDM process by ensuring that women have early access to high quality information about different aspects of treatment. While physicians and women had many shared views of how physicians involved women in TDM, there were also important differences which have implications for clinical practice and for the design of physician training programs.

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P47-13: COGNITIVE-AFFECTIVE FACTORS ASSOCIATED WITH UPTAKE OF, AND ADHERENCE TO, LYMPHEDEMA SYMPTOM MINIMIZATION PRACTICES IN BREAST CANCER SURVIVORS

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Approximately 20%–30% of women develop lymphedema (LE) following breast cancer treatment. Effective symptom management requires that women recognize early signs of lymphedema, and maintain precautionary practices over time. Data indicate that knowledge and use of symptom minimization precautions are poor. Little is known about how breast cancer survivors perceive their LE risk, and the cognitive-affective factors that promote the uptake and adherence to LE symptom minimization precautions. Guided by the Cognitive-Social Health Information Processing (C-SHIP) model, we conducted a longitudinal study of LE symptom-free women who are in remission following sentinel or axillary node surgery for Stages I-IIIa, primary breast cancer (N = 104) to assess barriers and facilitators associated with knowledge and adherence to LE symptom-minimization practices among breast cancer survivors. Specific aims included: (1) To delineate the underlying cognitive-affective mediating mechanisms that facilitate or undermine the uptake of LE symptom-minimization practices, and their sustained adherence over time; (2) To assess the moderating role of stable differences (i.e., monitoring attentional style) in the individual's cognitive-emotional profile on the uptake and adherence of LE symptom minimization practices over time. Preliminary data suggest that while a number of women are aware of LE minimization practices and their potential benefits, they are not incorporating all of the suggested minimization practices into their daily lives, especially those that may constitute active strategies. Moreover, our early findings suggest that promoting the maintenance of LE preventive/minimization behaviors and enhancing the management of LE risk-related emotions over time may be a worthwhile focus for a subset of individuals. Taken together, our preliminary findings support the importance of this study in increasing LE-related knowledge and improving health behaviors to reduce women's risk for developing LE. Through systematic investigation, we are developing a profile of the role of cognitive-emotional processing in the management of lymphedema. These data will ultimately be used to design and evaluate enhanced management protocols, tailored to the individual's cognitive-emotional signature.

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QUALITY OF LIFE

Poster Session P48

P48-1: A LONGITUDINAL STUDY OF HOT FLASHES AMONG BREAST CANCER SURVIVORS

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Few longitudinal studies of hot flashes (HtFs) among women with breast cancer have been published. We studied the experience of HtFs among breast cancer survivors from the University of Pennsylvania's Rowan Clinic following the first clinic appointment for 18 months. The 176 participants were primarily Caucasian, married, college educated, and working full-time. At baseline assessment, 51% were postmenopausal and 36% reported experiencing HtFs in the previous year. Following baseline, the onset of HtFs occurred in 42%. At the first report of HtFs, the most common physical symptoms were heat (83%), sweat (83%), and flushing (63%), and the most common feelings during HtFs were irritation (36%), frustration (20%), and anxiety (16%). The average number of HtFs experienced yesterday was 1.6 ($SD = 2.4$) and ranged from 0 to 10. On 11-point scales, average HtF severity, bother, and interference with daily life respectively were 4.2 ($SD = 2.2$), 4.0 ($SD = 2.5$), and 1.5 ($SD = 1.8$). Thirty-eight percent were using HtF treatment strategies, the vast majority of which were not prescription medications. Treatment was rated moderately effective ($M = 4.5$; $SD = 3.3$). A significantly greater proportion of women, who first reported HtFs at baseline, used treatment strategies while more women, who reported HtFs post-baseline, reported feeling frustration during hot flashes. A follow-up assessment 6- or 9-mo after the first report of HtFs was completed by 113 survivors, 87% of whom were experiencing HtFs. Although HtF frequency significantly increased, severity and bother decreased among women who reported HtFs at baseline. No significant changes were found for women with post-baseline HtFs, but compared to the former, these women reported significantly less daily interference and greater perceived treatment effectiveness. These data suggest that hot flash experiences among breast cancer survivors may be affected by pretreatment factors.

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P48-2: REDUCING THE BURDEN OF BREAST CANCER

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Introduction: This study investigates the effectiveness of a psycho-educational intervention with African American and Latina breast cancer survivors (BCS).

Methods: Participants were recruited from the California Cancer Registry (CCR), hospitals, community health clinics, and BCS support groups. Eligible participants in this study included women 18 years of age and older, within 1 to 6 years of being diagnosed with breast cancer, diagnosed with stages 0-III, not diagnosed with another type of cancer. The Functional Assessment of Cancer Therapy-Breast (FACT-G) was utilized to assess overall HRQOL. The FACT-G generates subscale scores on four dimensions of HRQOL (physical, social/family, emotional, and functional wellbeing). Based on their responses to the pre-test measure, participants were assigned to either the low intensity (LoTx) or high intensity (HiTx) treatment condition and completed both a pre-test measure and a post-test measure.

Results: The preliminary results of a total of 110 BCS (44 African American, 66 Latinas) are presented. Fifty eight BCS (53%) reported that they were diagnosed at Stage I, followed by 34 (31%) who were diagnosed at Stage II, and 17 (16%) who were diagnosed at Stage III. Participants in the LoTx group displayed slightly higher QOL scores during the pre-test ($M=62.7$, $SD = 8.9$) than the post-test ($M=61.8$, $SD=10$). Participants in the HiTx group showed an increase in QOL scores between the pre-test ($M=53.5$, $SD=5.9$) to the post-test ($M=57.1$, $SD=7.2$). At the baseline, there were statistically significant differences in both the LoTx and HiTx groups in overall QOL scores ($t=-6.49$, $p=.0001$). Independent sample t-tests were also conducted to assess whether differences exist in overall QOL based on ethnicity during the pre and post-test measure. Findings revealed that African American BCS reported higher QOL scores than Latina BCS during both the pre-test ($t=3.3$, $p = .001$) and the post-test ($t=2.3$, $p=.024$).

A repeated measures analysis was conducted to determine if the HiTx had an effect on overall quality of life as measured by the FACT-G after controlling for the baseline differences. As expected, a significant difference was found for overall QOL by study condition such that QOL scores improved from the baseline ($M=53.5$, $SD=5.9$) to approximately 3 months ($M=57.1$, $SD=7.2$) for participants in the HiTx group. However, the LoTx group did not show any significant change in the overall QOL score. Further, ethnicity did not significantly influence the amount of change in the overall QOL score ($F=.861$, $p=0.356$).

The qualitative results indicate that HiTx participants found the stress management component of the HiTx to be particularly helpful. Participants also reported increases in their confidence level in being able to communicate their needs to their medical providers after taking part in the intervention.

Discussion: Preliminary findings on the efficacy of the psychosocial intervention reveal significant improvements in HRQOL as measured by the FACT. Further, results

indicate that Latina BCS experience less favorable overall QOL than African American BCS.

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P48-3: WOMEN'S ATTRIBUTIONS OF MOOD AND COGNITIVE CHANGES FOLLOWING PROPHYLACTIC OOPHORECTOMY

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Women at elevated risk for hereditary breast and ovarian cancer who undergo prophylactic oophorectomy (PO) face physical and psychosocial changes more bothersome than those in natural menopause. Research has focused on body image and feelings of lost femininity following PO. However, negative affective and cognitive difficulties have been reported, as have positive changes such as decreased anxiety. It is unclear, however, whether these changes in affective and cognitive experience are attributed to alterations in hormone functioning, risk of cancer, or other factors. Understanding attributions for changes is important, as these influence satisfaction with decisions to undergo PO as well decisions about symptom management. We examined attributions of cognitive and affective changes among women who had undergone PO via content analysis of more than 5,000 postings on an internet-based discussion board (FORCE). Discussions of changes in mood and cognition since PO were categorized according to valence and attribution of change, yielding 141 unique postings. Results indicated that women posted about as often concerning positive mood and cognition changes (38%) as negative changes (43%). However, regardless of whether changes were positive or negative, they were 3.2 times more likely to be attributed to hormonal causes than nonhormonal causes. Among postings by women reporting positive changes, 83% attributed this to hormone replacement or nonhormonal causes (e.g., exercise or antidepressants), while among women reporting negative changes, 51% were attributed to hormone ablation. While women were about equally likely to attribute positive (72%) or negative (69%) changes to hormonal effects, negative experiences were more often attributed to loss of hormones (44%) than were positive experiences (17%). Women who experience negative changes following PO may consider utilizing contraindicated hormone replacement to obtain more positive outcomes.

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P48-4: ABSTRACT WITHDRAWN

P48-5: ABSTRACT WITHDRAWN

P48-6: ABSTRACT WITHDRAWN

P48-7: PRE-OPERATIVE ASSESSMENT ENABLES EARLY DIAGNOSIS AND SUCCESSFUL TREATMENT OF LYMPHEDEMA

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Background: Incidence of breast cancer (BC) related lymphedema (LE) ranges from 7-47%. Successful management of LE relies on early diagnosis using sensitive measurement techniques. We demonstrate the effectiveness of a surveillance program including pre-operative limb volume measurement and interval post-operative follow-up to detect and treat subclinical LE.

Methods: LE was identified in 43 of 196 women participating in a prospective, breast cancer morbidity trial. Limb volume was measured pre-operatively and at 3-month intervals following surgery. If a >3% increase in upper limb (UL) volume developed compared to pre-operative volume, a diagnosis of LE was made and a compression garment intervention was prescribed for 4 weeks. Upon reduction of LE, garment wear was continued only during strenuous activity, with symptoms of heaviness or with visible swelling. Subjects returned to the 3-month interval surveillance pathway. Statistical analysis was a repeated measures ANOVA by time and limb ($p \leq 0.001$) comparing the LE cohort with an age-matched control group.

Results: Time to onset of LE averaged 6.9 months post-operatively. The affected limb mean volume increase was 83 ml ($STD \pm 119$) ($6.5\% \pm 9.9$) at LE onset ($p = 0.005$) as compared to baseline. Following intervention, a statistically significant 48 ml (± 103) ($4.1\% \pm 8.8$) mean volume decrease was realized ($p < 0.0001$). Mean intervention duration was 4.4 weeks (± 2.9). Volume reduction was maintained at an average follow-up of 4.8 months (± 4.1) after intervention.

Conclusions: A short trial of compression garments effectively treats subclinical LE.

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DRUG DISCOVERY AND DEVELOPMENT II

Poster Session P49

P49-1: A NOVEL CLASS OF LIGAND-DERIVED ErbB RECEPTOR ANTAGONISTS

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The EGF family hormone NRG2 β potently stimulates ErbB4 tyrosine phosphorylation, whereas the NRG2 α splicing isoform has a lower affinity for ErbB4 and does not potently stimulate ErbB4 phosphorylation. The K45F mutation increases the affinity of NRG2 α for ErbB4 and permits potent stimulation of ErbB4 tyrosine phosphorylation. However, here we demonstrate that NRG2 β stimulates ErbB4 coupling to IL3 independence, but NRG2 α /K45F does not. Indeed, NRG2 α /K45F competitively antagonizes NRG2 β stimulation of ErbB4 coupling. Leu43 of NRG2 α /K45F is critical for antagonistic activity, as the NRG2 α /L43Q/K45F mutant stimulates ErbB4 coupling. Moreover, the analogous NRG2 β /Q43L mutant potently stimulates ErbB4 phosphorylation but fails to stimulate ErbB4 coupling and competitively antagonizes wild-type NRG2 β stimulation of ErbB4 coupling. NRG2 β stimulates Akt phosphorylation but NRG2 β /Q43L does not. Furthermore, PI3K activity is required for NRG2 β stimulation of ErbB4 coupling. This suggests that differential stimulation of ErbB4 coupling to the PI3K/Akt pathway is responsible for the functional differences between NRG2 β and NRG2 α .

Wild-type NRG2 β is a low-affinity agonist for EGFR/ErbB1. In contrast, in a number of model systems NRG2 β /Q43L fails to stimulate EGFR coupling to cell proliferation and competitively antagonizes EGF stimulation of EGFR coupling to cell proliferation. Similarly, the EGF/Q43L mutant behaves as a partial agonist with respect to EGFR coupling to cell proliferation and behaves as a partial competitive antagonist of EGF and amphiregulin stimulation of EGFR coupling to cell proliferation. We are currently evaluating the mechanisms by which NRG2 β /Q43L and EGF/Q43L competitively antagonize ligand-induced EGFR coupling to cell proliferation.

Thus, these data are the first evidence that an EGF family hormone may function as an antagonist of ErbB receptor coupling and of the interconversion of an ErbB receptor agonist and antagonist. Deregulated signaling by ErbB receptors and their ligands play important roles in human malignancies. Our data suggest that characterization of these roles must account for the possibility that either overexpression of receptor agonists or underexpression of receptor antagonists may contribute to ErbB receptor coupling to malignant phenotypes. Moreover, our data suggest that naturally occurring, context-dependent ErbB receptor antagonists and loss-of-efficacy point mutants of receptor agonists both hold potential as cancer chemotherapeutics.

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P49-2: SELECTIVE ABLATION OF TUMOR-ASSOCIATED MACROPHAGES SUPPRESSES METASTASIS AND ANGIOGENESIS

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Primary and metastatic neoplastic development both depend on the tumor microenvironment. Tumor growth requires angiogenesis, and the tumor-associated macrophages (TAMs) are key producers of growth factors, such as VEGF, that induce angiogenesis and support tumor cell survival. Legumain is an asparaginyl endopeptidase that is specifically overexpressed on the surface of endothelial cells and TAM in tumor stroma in addition to the neoplastic cells. Using a doxorubicin-based prodrug specifically activated in the tumor stroma by legumain, we showed that effective killing of both TAMs and endothelial cells lead to apoptotic death of these cells. Massive tumor cell death followed the death of endothelial cells and TAMs. The subsequent collapse of tumor microvasculatures resulted in complete tumor growth inhibition without any apparent toxicity. Targeting resident cells in the tumor microenvironment has distinctive advantages since both endothelial cells and TAMs are nontransformed and much more sensitive to chemotherapeutic agents versus tumor cells that are frequently multidrug resistant. The prodrug treatment effectively reduced TAMs in tumors and resulted in significant reduction in angiogenic factors and other growth factors that support tumor cell survival. The antiangiogenic effect is demonstrated by reduced vessel density in treated tumors. Consequently, the prodrug therapy exerts combined antiangiogenic and antitumor effect. The targeted prodrug activation permits metronomic dosing at an effective level critical to prevent the recovery of TAMs and endothelial cells; it effectively seizes the therapeutic windows created by normalization of tumor vasculature following angiogenic factor depletion. Consistent with the importance of TAMs in angiogenesis tumor progression, the accumulation of TAMs proceeds apparent angiogenic responses at the site of metastasis. More importantly, administration of this prodrug significantly reduces metastasis in metastatic models. The prodrug suppresses spontaneous metastasis as well as metastasis following surgical removal of primary tumors, a clinically relevant setting, and extends survival of the host without toxicity. Our findings indicate TAMs play a critical role in tumor development and metastasis.

The potent in vivo efficacy suggests that metronomic dosing of legumain-activated prodrug represents a novel anticancer strategy targeting multiple steps during tumor metastasis and angiogenesis.

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P49-3: NOVEL INHIBITORS OF THE EphB4 RECEPTOR AS ANTI-CANCER AGENTS

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The EphB4 receptor tyrosine kinase has recently emerged as a promising drug target in breast cancer. EphB4 is upregulated in breast cancer cells and promotes tumor angiogenesis (leading to increased tumor growth) by binding to its ligand, ephrin-B2, which is present in tumor blood vessels. Furthermore, inhibiting the binding of EphB4 to ephrin-B2 dramatically reduces tumor growth in animal models. However, the only inhibitor used so far is a soluble form of the extracellular portion of the EphB4 receptor, which is very large and difficult to prepare.

We have recently identified short peptides and small molecules that block binding of the EphB4 receptor to the ephrin-B2 ligand. The biological activity and structure-function relationship of these novel inhibitory molecules have been determined. In this presentation, we describe the application of the latest techniques in structure-based drug design and medicinal chemistry to develop novel inhibitors to study Eph receptor biology and develop new anticancer agents.

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P49-4: DEVELOPMENT OF 1- α -HYDROXYVITAMIN D5 AS A POSSIBLE CHEMOTHERAPEUTIC AGENT

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A non-toxic analog of vitamin D3, 1 α -hydroxy-24 ethyl-Vitamin D3 (1 α (OH)D5), was synthesized and evaluated for its efficacy against breast cancer cells. The results showed that the analog was antiproliferative in VDR and ER-positive breast cancer cells. In vivo studies using mammary carcinogenesis models, we observed that 1 α (OH)D5 inhibited both MNU- and DMBA-induced mammary carcinogenesis at nontoxic concentrations. Moreover, the analog was tolerated at 10-fold higher concentration as compared to 1,25(OH)2D3 in rats. The effect of 1 α (OH)D5 is mediated by VDR. Confocal microscopic analysis has shown that VDR co-localizes with 1 α (OH)D5, indicating that its action is mediated via VDR. However, the binding affinity of 1 α (OH)D5 to VDR was lower than that of 1,25(OH)2D3. It is not clear whether the agent needs to be metabolized for its action. The mechanism of action of 1 α (OH)D5 has been reported in various publications. Based on these results, we hypothesized that 1 α (OH)D5 administered at a non-toxic concentration to women with breast cancer will induce differentiation and prevent progression of malignancy. We completed 30-day preclinical toxicity studies in rats and dogs under GLP regulations. The results showed rats tolerated 1 α (OH)D5 at a higher concentration than dogs. Based on the extensive preliminary results and preclinical toxicity and stability studies, an IND application was submitted to the FDA for approval. However, due to the shortcomings of the stability studies it was not approved. More recently, additional stability studies were carried out using 1 α (OH)D5 prepared under GMP conditions and the IND is now approved by the FDA for Phase 1 clinical trials for breast cancer patients. A multicenter clinical trial will be initiated as soon as an appropriate formulation is developed.

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P49-5: NEOSERGIOLIDE – THE FIRST POTENT BI-FUNCTIONAL NATURAL PRODUCT THAT SIMULTANEOUSLY TARGETS STAT3-DEPENDENT BREAST TUMORS AND TUMOR HYPOXIA

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Background and Objectives: In response to hypoxic conditions, the transcription factor hypoxia-inducible factor 1 (HIF-1) up-regulates genes that activate angiogenesis, glycolytic metabolism, metastasis, drug resistance, and others that promote hypoxic adaptation, survival, and malignant progression. In clinical samples, over-expression of the oxygen regulated HIF-1 α subunit is associated with advanced disease stages and

poor prognosis in breast cancer. Since HIF-1 is inactive in normal cells, HIF-1 inhibitors represent a new generation of tumor-specific agents with minimal effects on normal tissues. Constitutive activation of signal transducer and activator of transcription 3 (STAT3) has recently been shown to be associated with abnormal growth, inhibition of apoptosis, and an overall increase in the malignant progression of breast tumors. Both STAT3 and HIF-1 have emerged as major molecular targets for breast cancer drug discovery and certain target genes such as VEGF are regulated by both STAT3 and HIF-1. Over 5000 crude plant extracts were evaluated in an oncogenically transformed rat fibroblast NA/Fr3T3 cell-based reporter assay for novel STAT3 inhibitors. The unusual quassinoid neosergiolide was isolated from an active extract of the Amazonian plant *Picrolemma sprucei*. Neosergiolide also potently inhibited hypoxia-induced HIF-1 activation in T47D breast tumor cells. The objectives of this research are: (1) characterize the potent STAT3/HIF-1 inhibitory activity of neosergiolide in breast tumor cells; (2) examine the effects of neosergiolide on STAT3 and HIF-1-regulated breast tumor cell proliferation/survival, angiogenesis, and invasion/metastasis; and (3) determine if other structurally related quassinoids have a similar capacity to concurrently inhibit both breast tumor cell survival and stress adaptation pathways regulated by STAT3 and HIF-1.

Methodologies: A panel of established human breast tumor cell lines that represent different disease stage, ER status, hypoxia responsiveness, and STAT3 dependence are used as in vitro models. Concentration-response studies will be performed to determine the effects of neosergiolide on STAT3 and HIF-1 activity using cell-based reporter assays. The outcomes of neosergiolide treatment on the expression of representative STAT3 and HIF-1 target genes will be examined at both mRNA and protein levels. A three-tiered approach will be employed to determine the effects of neosergiolide on: (a) STAT3-mediated and hypoxic breast tumor cell proliferation/viability; (b) angiogenesis in vitro; and (c) breast tumor cell migration and invasion. Normal human mammary epithelial cells will be employed as in vitro models to assess the cytotoxicity.

Results: Neosergiolide inhibited both hypoxia (1% O₂)- and 1,10-phenanthroline-induced HIF-1 activation in T47D cells. Hypoxic induction of secreted VEGF protein, a potent angiogenic factor, was inhibited by neosergiolide. In T47D cells, neosergiolide inhibited hypoxic induction of HIF-1 α protein without affecting the constitutively expressed HIF-1 β protein. Among the breast tumor cell lines examined, MDA-MB-231 cells appear to be the most sensitive to the growth inhibitory effect of neosergiolide.

Conclusions: Neosergiolide is a new HIF-1 inhibitor. Upon completion of the proposed objectives, we will be able to gain more knowledge regarding the therapeutic potential of neosergiolide for breast tumor, as well as its mechanism of action.

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P49-6: INHIBITING THE FOLDING AND MATURATION OF HER2 AND ER USING A NOVEL CLASS OF Hsp90 INHIBITORS

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HER2 and ER play critical roles in breast cancer tumorigenesis and disease progression. It has been demonstrated that there are extensive interactions between the signaling pathways of the HER2 and ER receptors, suggesting that targeting both receptors simultaneously may be an effective route to anti-breast cancer therapeutics. One way to achieve this is to prevent the proper folding and maturation of HER2 and ER by targeting its upstream chaperone proteins, such as heat shock protein 90 (Hsp90). Hsp90 is a highly abundant molecular chaperone that is responsible for the proper folding, maturation and activation of many oncogenic proteins including HER2 and ER. Hsp90 has been validated as an anti-cancer drug target. Proper Hsp90 function requires its ATPase activity and its interaction with different cochaperones. Current Hsp90 inhibitors act by inhibiting the ATPase activity of Hsp90 (e.g., 17-AAG), leading to the destabilization and degradation of Hsp90 client proteins. Here we present our research identifying and characterizing a novel class of Hsp90 inhibitors by disrupting its interaction with an essential cochaperone called heat shock organizing protein (HOP). We have developed the very first high-throughput in vitro screening assay using a beads-based Amplified Luminescence Proximity Homogenous Assay (ALPHA) technology to identify compounds that can specifically disrupt the Hsp90-HOP interaction. After screening over 100,000 compounds using this assay, 6 compounds in the toxoflavin structural class have been identified as positive hits that can disrupt the interaction in vitro with IC₅₀ values in the micromolar range. When tested in cultured breast cancer cells overexpressing HER2, these compounds were shown to greatly down regulate the total and phosphorylated protein levels of HER2 using western blotting. More significantly, this new class of inhibitors, in contrast to 17-AAG, do not induce the expression levels of another ubiquitously expressed chaperone protein, Hsp70. Hsp70 is a well-known antiapoptotic protein and plays critical roles in cell survival; overexpression of Hsp70 is one of the main mechanisms underlying drug resistance developed in response to 17AAG inhibition of Hsp90. The impact of these compounds on breast cancer research is threefold. First, these represent conceptually a new strategy by which to inhibit HER2 and ER function since they will never reach their matured functional state, while current breast cancer therapies use antagonists for ER and/or HER2 to inhibit the activity of the already overexpressed mature proteins. Second, these compounds can be used

as chemical probes that help dissect the complex Hsp90-cochaperone interaction network that will lead to a better understanding of the detailed molecular function mechanism of Hsp90 for better future inhibitor development. Third, these compounds offer great potential to be developed into novel breast cancer therapeutics without incurring the drug resistance by Hsp70 overexpression, either used alone or in combination with other known drugs.

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P49-7: MODULATING THE FUNCTION OF ONCOGENIC p53 IN THE INTACT ORGANISM USING SMALL MOLECULES TO MANIPULATE PROTEIN FOLDING

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Mutations in the p53 tumor suppressor protein are highly common in breast cancer. The conformation of wild-type p53 protein is intrinsically flexible, and cancer-related mutations reduce its stability leading to its misfolding and malfunction. Misfolding of p53 is reversible in vitro and in cultured cells under certain experimental conditions. This suggests that the modulation of p53 folding and function using small molecules that can be readily synthesized and easily delivered is a promising therapeutic approach. However, so far, no compound that directly binds and modulates p53 core domain was found possibly due to a lack of well-defined clefts for binding in the p53 protein surface. Chemical chaperones are small molecules that can stabilize protein structure through solvent-mediated interactions. Chemical chaperones, such as trimethylamine-N-oxide (TMAO), glycerol, and D₂O were capable of correcting folding of mutant p53 in vitro accompanied by its reactivation in cell lines. However, these studies have not been extended to the living animal.

Objective: Develop an experimental system for screening for compounds restoring folding and function of mutant p53 proteins in the living organism. The fruit fly, *Drosophila*, is especially suitable for screening candidate molecules for their restorative effect in the intact organism. The fly p53 (Dmp53) is highly homologous to its human counterpart both structurally and functionally. When overexpressed, the wild-type Dmp53 causes overt defects comparable to p53-derived human tumors. Importantly, amino acid replacements in Dmp53 corresponding to cancer-associated substitutions in p53 are conserved in Dmp53. Two of them were examined and were shown to exert dominant-negative (DN) phenotypic effect comparable to the DN effect of mutant p53 in humans. In flies, this DN effect leads to easily score-able phenotypes involving eye morphology and longevity.

Experimental Plan: (1) Generate and express mutant Dmp53 proteins carrying amino acid substitutions corresponding to oncogenic mutations in human p53. (2) Expose the purified proteins, and wild-type Dmp53 as a control, to increasing concentrations of potential chemical chaperones in vitro; examine their effect on folding of the proteins using biophysical methods and on their DNA binding ability. Promising compounds will be assayed in vivo (see: 4). In parallel: (3) Generate fly strains overexpressing these mutant versions of Dmp53 and verify their ability to cause the DN phenotypes as reported. (4) Feed larvae and adults exhibiting the DN phenotypes with increasing concentrations of promising test compounds in the medium. Wild-type *Drosophila* as well as strains overexpressing either normal or previously reported mutant Dmp53 will serve as a control. Ability of the tested compounds to counteract the DN phenotypic effects of the tumor-associated versions will be monitored. We shall verify that the effect of the compound is p53-specific using an available p53 specific reporter. Untreated individuals of all the above genotypes will serve as reference. We plan reiteration of cycles of testing potential compounds followed by rational design of and retesting of the improved analogues, aiming at enhanced efficacy. The best leads will be further developed using de novo synthesis of putative chaperones.

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P49-8: TARGETING ESTROGEN RECEPTOR SIGNALING VIA INTERFERENCE WITH ENZYMATIC ACTIVATION OF A CRITICAL RNA COACTIVATOR BY A NOVEL TYPE OF RNA INHIBITOR

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Background and Objectives: Estrogen receptor (ER) α is a major contributor to early-stage estrogen (E²)-dependent breast carcinogenesis and ER-antagonist drugs such as tamoxifen (Tam) are widely used in hormonal therapy. However, alternative pathways that block ER α -activity are urgently needed because current drugs may not be fully effective or result in unwanted side effects. Coactivators are critical components of ER-dependent gene expression. One striking coactivator is steroid receptor RNA activator (SRA), which functions as a noncoding RNA. SRA greatly stimulates ER activity and is overexpressed in ER⁺ breast cancers, suggesting that SRA contributes to breast car-

cinogenesis. SRA is posttranscriptionally modified by pseudouridine synthases (Pus), a process required for SRA coactivator function and ER-activity. In particular, Pus1p isomerizes uridine (U) to pseudouridine (ψ) in SRA in multiple positions, and pseudouridylation of U206 in hairpin STR5 is especially critical (see Figure). Thus, Pus1p contributes to an ER-activating pathway that is independent from antagonist-targeted pathways. We hypothesized that a synthetic, 61 nt STR5 RNA fragment inhibits Pus1p-activity, modification of SRA, and thereby ER-signaling. The objectives of this study are (1) to establish that STR5, and possibly higher potency derivatives, inhibit ER-dependent signaling in MCF-7 cells, (2) to establish that STR5+Tam suppresses ER-activity more potently than either agent alone, and (3) to confirm that STR5 inhibits activity of Pus1p.

Methodologies: Effects of transfected STR5 on ER-signaling are analyzed by measuring activity of a cotransfected, ER-dependent reporter plasmid and by measuring ER-dependent growth of MCF-7 cells. We will also determine if higher potency derivatives of STR5 can be designed. The same assays are used to establish if combination therapy of STR5+Tam results in additive or synergistic inhibition of ER-activity. Effects of STR5 on Pus1p-dependent pseudouridylation of SRA are established in vitro in a tritium-release assay.

Preliminary Results: STR5 strongly inhibited E²-dependent transactivation of reporter gene activity in a dose-dependent fashion whereas cotransfection with a Pus1p-expression vector counteracted this effect. Furthermore, STR5 inhibited Pus1p-dependent pseudouridylation of SRA in a dose-dependent manner in vitro.

Conclusions: Targeting of this novel coactivator pathway with STR5-derived RNA could be an important new strategy to inhibit ER-signaling that is independent from ER antagonist therapies. If confirmed, the benefits of this method, alone or in combination with ER-antagonists could have a significant impact on breast cancer patient care.

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P49-9: A MOLECULAR SWITCH OF HER2: TARGET FOR RECEPTOR HETERODIMERIZATION

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A number of treatment strategies are currently being used to target the HER2 (erbB-2) receptor in breast carcinomas, including monoclonal antibodies directly against its extracellular domain (ECD) and small molecules blocking its tyrosine kinase activity. Yet, there is a need to develop novel therapies inhibiting HER2 dimerization with other HER family members, not necessarily in the context of HER2 overexpression. To test the hypothesis that HER2 activation might occur through a functional site at the ECD responsible for HER2 homo- and hetero-dimerization, we introduced a series of deletion mutations into the third sub-domain of HER2 ECD, which contributes most of the determinants involved in ligand binding and signal transduction. The HER2 ECD deletion mutants were expressed in MCF10A untransformed breast epithelial cells; SKBr-3 breast cancer cells naturally bearing HER2 gene amplification and HER2 protein overexpression; and in MCF-7/Her2-18 cells. The smallest deletion in the ECD domain of HER2 (HER2/ Δ 6) was sufficient to disrupt the oncogenic signaling arising from HER2: (1) overactivation of HER2, HER1, MAPK, and AKT was observed in MCF10A/HER2 and failed to occur in cells expressing the HER2 deletion mutant MCF10A/ Δ 6. (2) Expression of HER2/ Δ 6 prevented HER2 Y1248 phosphorylation via ligand induced HER1 and HER3 heterodimerization. (3) MCF10A/ Δ 6 cells did not form branching and morphogenesis, as the MCF10A/HER2 cells do, and form ascini as the wild-type MCF10A cells. (4) HER2 Δ 6 failed to promote anchorage-independent soft-agar colony formation and Taxol resistance as wild-type HER2 did in MCF10A cells. (5) Expression of HER2/ Δ 6 significantly reduced anchorage-independency and enhanced Taxol efficacy in HER2-overexpressing cells.

Conclusions: These findings reveal for the first time that an essential activating sequence exists in the sub-domain III of the HER2 ECD as its disruption disables the HER2 homo- and hetero-dimerization loop, blocks subsequent activation of the HER2-driven oncogenic signaling, and generates a dominant-negative form of HER2. We are currently testing high affinity peptides developed with specificity for this molecular-activation switch. These compounds may represent a novel targeted approach for the

management of breast carcinomas escaping HER2 treatment and requiring the therapy to target receptor heterodimerization.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0686.

P49-10: SODIUM-POTASSIUM ATPase INHIBITORS: A COMPLETELY NEW STRATEGY FOR TREATING BREAST CANCER BY POTENTLY SUPPRESSING HYPOXIA-INDUCED TUMOR CELL SURVIVAL PATHWAYS WITH EXTREMELY LOW DOSES OF CARDIAC GLYCOSIDES

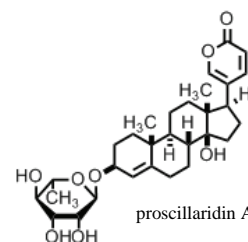
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Background: Epidemiological studies and the most recent laboratory research suggest that digitalis treatment may suppress breast cancer. Digitalis, ouabain, and other cardiac glycosides inhibit sodium/potassium-ATPase (Na^+, K^+ -ATPase) and have long been used to treat cardiovascular disorders. Recently, Na^+, K^+ -ATPase inhibitors, such as ouabain, were shown to induce apoptosis, disrupt mitochondrial function, act as antagonists to the estrogen receptor (ER), and suppress the growth of ER-negative breast tumor cells, at nM concentrations below the level required to significantly inhibit the Na^+, K^+ -ATPase. We discovered that ouabain inhibited the hypoxic induction of hypoxia-inducible factor-1 (HIF-1) activation in human breast tumor T47D cells at low nM concentrations. The objective of this study is to test whether HIF-1 inhibition represents a novel molecular mechanism (and potential therapeutic application) for ouabain and related cardiac glycosides that may be used to treat breast cancer.

Methods: This research included the following:

(1) characterizing the potent HIF-1 inhibitory activity of ouabain in a panel of human breast tumor cell lines that represent different disease stages, ER status, hypoxia responsiveness, and sensitivity to cardiac glycosides in vitro; (2) examining the effects of ouabain on hypoxic breast tumor survival, angiogenesis, and invasion-metastasis; and (3) determining if other cardiac glycosides have HIF-1 inhibitory activities comparable to that of ouabain.



Results: Ten structurally related cardiac glycosides were evaluated for their effects on HIF-1 activation in three breast tumor cell lines. The cytotoxic effects of these compounds were also examined. Ouabain, digitoxin, digoxin, and the two buf-type cardiac glycosides bufalin and proscillaridin A were found to be potent inhibitors of hypoxia-induced HIF-1 activation in two different breast tumor cell lines (IC₅₀ values 0.012 – 0.257 μM , T47D cells; IC₅₀ values 0.016 – 0.21 μM , MCF-7 cells).

Conclusions: Proscillaridin and other cardiac glycosides potently inhibit hypoxia-induced gene expression in T47D breast tumor cells. The MDA-MB-231 tumor cell line is traditionally used as a model for highly mutated and aggressive breast tumors. The apparent heterogeneous nature of the MDA-MB-231 cell line precluded its use as a model for HIF-1 regulation. Cardiac glycosides that modulate Na^+, K^+ -ATPase may exhibit unusual effects on cell viability assays that may provide insight into recent reports of the antitumor properties of these agents.

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P49-11: NANOSPHERIC CHEMOTHERAPEUTIC AND CHEMOPROTECTIVE AGENTS

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Nanotechnology can have a profound effect on the delivery of therapeutics by improving their stability, circulation times in the body, permeability through cell membranes and targeting of affected cells. Paclitaxel (Taxol[®]) is used widely for the treatment of breast and other types of solid tumor cancers.¹ Paclitaxel is only sparingly soluble in water and its intravenous administration depends on the use of Cremophor[®] EL (polyethoxylated castor oil) to obtain a therapeutically effective solution. Unfortunately, use of Cremophor increases patient toxicity and can lead to clinically important adverse effects, including acute hypersensitivity reactions and peripheral neuropathy.²

To improve the therapeutic potency of paclitaxel and to overcome the toxicities associated with Cremophor-paclitaxel, we have developed a nanosphere system based on biodegradable and non-cytotoxic copolymers.^{3,4} This system is intended to improve

delivery of drugs to tumors while eliminating the adverse side-effects of other drug carriers. The ABA-type triblock copolymers consist of poly(ethylene glycol) A-blocks and hydrophobic low molecular weight polyarylates B-blocks made of desaminotyrosyl-tyrosine alkyl esters (DTR) and diacids.³ These copolymers self-assemble into nanospheres with hydrodynamic diameters of ca. 70 nm. These nanospheres form stable complexes with a wide variety of hydrophobic therapeutics and deliver them efficiently to cells in vitro.⁴

Aim: To evaluate the potential of tyrosine-derived nanospheres as drug delivery vehicles; anti-tumor activity, pharmacokinetics and biodistribution of paclitaxel-loaded nanospheres in human breast tumor xenograft in mouse model.

Results: (1) Within the investigated range of nanosphere concentration (0.05 to 4 mg/mL), and copolymer compositions, no significant decrease of the cell metabolic activity of KB cervical carcinoma cells was detected, confirming that these nanospheres do not induce any short-term cytotoxicity. (2) These nanospheres provide substantially enhanced delivery of paclitaxel to KB human carcinoma cell in vitro. (3) The nanospheres alone and nanospheres-paclitaxel (dose of 15 mg/kg) do not exhibit any in vivo toxicity based on the absence of significant weight loss (< 15%), change in vital behavior, skin irritation and sensitization at the injection spot in any of the treated groups. (4) Tyrosine-derived nanospheres containing paclitaxel exhibit similar anti-tumor activity in a breast cancer xenograft model to that of clinically used formulation of Cremophor-paclitaxel. (5) PK studies revealed that nanospheres-paclitaxel in murine plasma have a larger volume of distribution, higher clearance, and longer half-life than Cremophor-paclitaxel.

The evaluation of relative efficacy and potential synergies of nanospheres containing both anti-tumor drugs and other chemotherapeutic and chemopreventative drugs such as analogues of vitamin D3 is currently under investigation.

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P49-12: FoxM1 INHIBITOR, THIOSTREPTON IS A POTENTIAL DRUG AGAINST BREAST CANCER

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The oncogenic transcription factor forkhead box M1 (FoxM1) is overexpressed in a number of different carcinomas, including breast carcinomas, whereas its expression is turned off in terminally differentiated cells. For this reason, FoxM1 is an attractive target for therapeutic intervention in cancer treatment. As a first step toward realizing this goal, in this study, we isolated the antibiotic thiazole compound thiostrepton as an inhibitor of FoxM1 transcriptional activity. Using chromatin immunoprecipitation experiments, we found that thiostrepton inhibits binding of FoxM1 to target promoters. Interestingly, we observed that thiostrepton was able to downregulate the transcriptional activity as well as the protein and mRNA levels of FoxM1. However, thiostrepton did not affect transcriptional activity of p53, TCF/Lef, or GLI, suggesting that it specifically targets FoxM1. Furthermore, we found that thiostrepton was able to efficiently inhibit the growth of human breast cancer cell lines with IC₅₀ between 1.6 and 3.5 micromoles. Treatment of human breast cancer cells with thiostrepton led to predominant downregulation of FoxM1 and apoptosis, suggesting that its proapoptotic activity may be linked to inhibition of FoxM1. Our data suggest that thiostrepton, which has been already been approved by the FDA for animal use, may have a potential for further clinical development against breast cancer.

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P49-13: DEVELOPMENT OF SPECIFIC SMALL MOLECULE INHIBITORS OF MATERNAL EMBRYONIC LEUCINE ZIPPER KINASE

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Among more than 500 protein kinases, many have been implicated in cancers. The serine/threonine kinases have recently attracted researchers' attention as promising anticancer targets for drug development. Maternal embryonic leucine zipper kinase (MELK) is a serine/threonine kinase, and while its function within both cellular and organism context remains obscure, a large variety of poorly differentiated aggressive cancers clearly benefit from elevated expression levels of this kinase. The mammary

tumors exhibit particularly elevated levels of MELK expression thus justifying selection of this kinase as a pharmacological target against this deadly disease. Recent advances in computational biology techniques allowed us to fill structural information gaps due to the absence of MELK crystal structure. We have exploited unique sequence features of the MELK activation loop by generating validated homology model of MELK catalytic domain in its putative inactive conformation. The virtual ligand screening allowed us to discover several specific MELK inhibitors with IC₅₀s ranging from 120 nM to 1.2 μM. The specificity of lead MELK inhibitors was further confirmed by screening against a diverse kinase panel composed of 70 most relevant kinases. The inhibitors were tested against several cancer cell lines with varying levels of MELK expression. All of our compounds efficiently inhibited cell proliferation through cell cycle arrest in G2/M phase. These observations are consistent with previously published data of RNAi experiments where G2/M cell cycle arrest has been reported in response to the abolishment of MELK protein levels.

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P49-14: IDENTIFICATION OF PROCASPASE-ACTIVATING SMALL MOLECULES THAT INDUCE DEATH SELECTIVELY IN CANCER CELLS

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A hallmark of cancer is resistance to apoptosis. Many cancers are able to resist pro-apoptotic signals due to mutations or aberrant expression levels of proteins in the apoptotic cascade. These cells thus evade apoptosis and can proliferate in an uncontrolled fashion. The "classic" apoptotic pathways converge in the activation of procaspase-3/-7 to caspase-3/-7; caspase-3 and caspase-7 are the key executioner caspases that catalyze the hydrolysis of scores of protein substrates.

A separate and less understood apoptotic pathway occurs in response to stress signaling. In this pathway, procaspase-2 is recruited and activated by a complex called the PID-Dosome, which contains death-domain-containing PIDD and adaptor protein RAIDD. Since some cancers (such as breast, lung, and prostate) are known to have elevated expression levels of procaspases, and inactivating caspase mutations in cancer are rare, a small molecule that activates procaspases could be a promising strategy to induce cell death selectively in cancer.

High-throughput screening (HTS) was thus utilized in search of compounds that could increase the activity of procaspase-2 and procaspase-7. For procaspase-2, this screen led to the discovery of two drug-like molecules with half-maximal activation concentrations (EC₅₀) of approximately 25 μM. Further derivatization of the hits failed to produce more potent procaspase-2-activating compounds. Furthermore, the procaspase-activating compounds were found to also activate other enzymes such as chymotrypsin and β-lactamase. Further experiments indicate that these molecules act in a surfactant-like manner by stabilizing the enzyme or relaxing the secondary structure to increase enzyme activity. Just as nonspecific inhibitors can be false hits in HTS, evidence will be presented in which general enzyme activators can also be nuisance hits in high-throughput enzyme activation screens.

Procaspase-7 may play an important role in some breast cancers; for example, procaspase-7 is thought to be the predominant procaspase in the caspase-3-deficient MCF-7 cell line. Therefore, the identification of procaspase-7-activating compounds is of great interest. Thus, an HTS campaign was conducted on ~175,000 compounds in search of compounds that would enhance the activity of procaspase-7. Several compounds that have this property have now been identified and validated, and the mode of in vitro procaspase-7 activation has been determined. The efficacy of these compounds in vitro and their ability to induce apoptosis in the HL-60 leukemia cell line and MCF-7 and MDA MB-231 breast cancer cell lines will be discussed. The activation of procaspases to induce cell death may be a potential tactic to selectively fight breast cancer, as well as other cancers.

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P49-15: G-QUADRUPLEXES INDUCE APOPTOSIS

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Several studies have demonstrated that G-quadruplex oligodeoxynucleotides (GQ-ODNs), which are capable of forming G-quadruplexes, exhibit antitumor activities both in tissue culture and in animal models of xenografted tumors. However, the molecular basis for their antitumor activity remains unclear. The long-term goal of this application is to develop G-quadruplex-based therapy for breast cancers. In the current study, we showed that a variety of telomeric G-tail oligodeoxynucleotides (TG-ODNs) exhibited antiproliferative activity against many tumor cells in culture. Systematic muta-

tional analysis of the TG-ODNs suggests that the antiproliferative activity depends on the G-quadruplex conformation of these TG-ODNs. TG-ODNs were also shown to induce PARP1 cleavage, PS flipping, and caspase activation, indicative of induction of apoptosis. TG-ODN-induced apoptosis was largely ATM (ataxia telangiectasia mutated)-dependent. Furthermore, TG-ODN-induced apoptosis was inhibited by the JNK inhibitor SP600125. Indeed, TG-ODNs were shown to activate the JNK pathway in an ATM-dependent manner as evidenced by elevated phosphorylation of JNK and c-Jun. Interestingly, a number of G-quadruplex ODNs (GQ-ODNs) derived from non-telomeric sequences also induced ATM/JNK-dependent apoptosis, suggesting a possible common mechanism of tumor cell killing by GQ-ODNs.

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P49-16: DISCOVERY OF NOVEL SMALL-MOLECULE INHIBITORS OF HER2/NEU: COMBINED LIGAND-BASED AND TARGET-BASED APPROACH

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Aberrant signaling involving HER receptors stimulate pathways that activate many of the properties associated with cancer, including proliferation, migration, metastasis, angiogenesis, and resistance to apoptosis. Due to the high frequency of abnormalities in receptor signaling in human cancers, the HER family is an attractive target for therapeutic development. The frequently observed causes of signaling abnormalities in cancers involving HER receptors are overexpression of receptors, overproduction of growth factor ligands, and ligand-independent receptor activation. The HER receptors are overexpressed or deregulated in a wide variety of cancers including breast, colorectal, ovarian, prostate, and non-small cell lung cancers. Importantly, overexpression of HER receptors is associated with poor disease prognosis and reduced survival. Mounting preclinical and clinical evidence supports the rationale behind the HER family targeted anticancer therapeutic approaches. In an effort to develop a quantitative ligand-binding model for HER2 tyrosine kinase, combined virtual library screening models were developed to identify novel sets of molecules with activity in HER2 over-expressing cells. A search of a 3D database containing 350,000 small molecules using these consensus models yielded 57 compounds. Seven compounds with significant activity in SKBr3 cell were selected for further preclinical studies.

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P49-17: ABSTRACT WITHDRAWN

P49-18: INHIBITION OF THE RhoC GTPase-MEDIATED INFLAMMATORY BREAST CANCER PHENOTYPE BY FARNESYL TRANSFERASE INHIBITORS

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Inflammatory breast carcinoma (IBC) is a highly aggressive form of locally advanced cancer that invades the dermal lymphatics of the breast. Due to its highly invasive and metastatic phenotype, IBC carries a poor prognosis with a 5-year disease-free survival rate of <45%. We have shown that RhoC GTPase is overexpressed in >90% of IBCs and identified RhoC as an oncogene involved in conferring the metastatic phenotype. RhoC is a small geranylgeranylated GTPase that reorganizes the cytoskeleton during cellular motility.

Farnesyl transferase inhibitors (FTIs) interfere with GTPase farnesylation and activity and can modulate the growth of Ras-transformed tumor cells. However, clinical use of FTIs on Ras-based tumors has proven disappointing. Recent focus has turned to non-Ras targets of FTIs. Farnesylated RhoB (fRhoB) is a putative non-Ras target of FTI action, leading to accumulation of geranylgeranylated RhoB (ggRhoB). We have shown that treatment of RhoC-overexpressing SUM149 IBC cells with the FTI L-744,832, leads to reversion of the RhoC-induced phenotype, including significant decreases in anchorage-independent growth, motility, and invasion. These in vitro attributes correlate with the malignant behavior of the IBC cells. FTI treatment increases RhoB levels without affecting total (GDP- and GTP-bound) and active (GTP-bound) levels of RhoC. ggRhoB transfection into SUM149 cells mimics the effects of FTI, suggesting that FTI action is mediated by ggRhoB. These observations are consistent with the findings of other studies performed in various transformed cell systems, including epithelial cells. On the basis of our observations, we hypothesized that FTI treatment will be effective at inhibiting progression of RhoC-overexpressing IBC tumors.

Akt is a serine/threonine phosphorylated molecule that effects tumor cell survival, growth, and motility in breast cancer cells. Rho GTPases contain a putative Akt phosphorylation site between the GTPase and effector-binding domains. However, only Rac1 has been studied with regard to Akt phosphorylation. Other reports suggest that

levels and cellular localization of active Akt1 (pAkt1) is mediated by FTI treatment and by RhoB.

We demonstrate that compared with non-IBC cell lines, the SUM149 expresses high levels of pAkt1 due to loss of Pten. In addition, in the IBC cells, RhoC GTPase is highly serine phosphorylated. Inhibition of Akt1, either by treatment with an inhibitor specific to Akt1 or through downregulation of Akt1 using an siRNA approach, leads to a significant decrease in IBC cell invasion without affecting cell viability.

Taken together, our data suggest that FTIs may affect Akt1 phosphorylation of RhoC leading to a highly metastatic cell. In addition, FTIs may be viable therapeutics to inhibit the IBC metastatic phenotype.

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P49-19: NOVEL AND EFFICIENT SYNTHESIS OF THE PROMISING DRUG CANDIDATE DISCODERMOLIDE

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Background and Objectives: The goal of this project is to develop an efficient synthesis of the microtubule-binding, antitumor antibiotic discodermolide. Because discodermolide is available only in minute quantities from the collection and extraction of a deep sea sponge, its development as a drug requires a supply from chemical synthesis. Syntheses published prior to our initiation of this project were unacceptably long.

Discodermolide has a 24-carbon chain that is decorated with methyl and oxygen substituents at 13 chiral centers. Retrosynthetic analysis of the discodermolide molecule invariably leads to three molecular building blocks that are to be linked in the final steps of the synthesis. These building blocks contain stereotetrad, stereotriad, and stereopentad stereochemical arrays.

Methodologies: Seeking a novel approach that would radically shorten the synthesis, we considered the Mother Nature's "chiral pool" as the source of a starting material that would give us the most complex of the three building blocks, the stereopentad. We recognized the C15-C21 stretch of discodermolide in the C1-C7 stretch of oleandomycin, a *Streptomyces* metabolite formerly marketed in the US as an agricultural antibiotic. Oleandomycin can be obtained in large amounts by industrial fermentation. Our plan was to excise a stereopentad-containing fragment from oleandomycin and then elaborate it to a stereopentad-containing diene, a known discodermolide intermediate.

Results to Date: A degradation scheme for oleandomycin was developed in order to effect four key transformations: migration of functionality from the 8-8a position to the 7-8 position, removal of the carbohydrate appendages, selective protection of the resulting C-2 and C-4 hydroxyl groups, and cleavage of the macrolide ring to afford a seven-carbon intermediate that could be converted to the desired diene. Successful procedures for these transformations as well as additional useful protocols that were discovered during our investigations are described in detail in a publication.

We next considered methods that might be exploited for the more efficient synthesis of the other half of the discodermolide molecule, a C-1 to C-14 equivalent. A common stereodiad building block, derived from the 2,3-Wittig rearrangement of the methallyl ether of a chiral allylic alcohol, gave stereotriad and stereotetrad building blocks. These were linked to give the second major intermediate required for our synthesis. These results are being reported in one published manuscript and one submitted manuscript.

Our synthesis and others as well would be significantly improved by the use of synthetic methods that would permit the introduction of "short-cuts." Two methods developed for this purpose are described in manuscripts in preparation.

Conclusions: A novel synthesis of discodermolide is on track. Our methods will contribute to the ability of chemists to prepare polyketide antibiotics and their analogs for biological and clinical testing. Furthermore, should discodermolide or another difficult-to-obtain polyketide be approved as a drug, the manufacture of large quantities will be facilitated by these methods.

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P49-20: DISCOVERY OF NOVEL HDAC INHIBITORS FOR THE TREATMENT OF BREAST CANCER: MAPPING NEW BINDING SITES OF HISTONE DEACETYLASE USING SMALL MOLECULES AS PROBES

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Histone deacetylases (HDACs) are a family of enzymes that regulate chromatin remodeling and gene transcription. They consequently control critical cellular processes, in

cluding cell growth, cell cycle regulation, DNA repair, differentiation, proliferation, and apoptosis. Chemical inhibitors of HDACs (HDACi) have been shown to inhibit tumor cell growth and induce differentiation and cell death. It is believed that HDAC inhibitors provide unique opportunities in the discovery of small molecule therapeutics for the treatment of cancer. A variety of natural and synthetic compounds have been reported to show HDAC inhibitory activity and antitumor effects. Multiple studies have shown that designing compounds that are selective for one or a limited number of specific HDAC isoforms may improve overall therapeutic efficacy and lower toxicity. While some rather limited degree of isoform selectivity has been obtained for a few compounds, the problem of identifying selective inhibitors is far from being solved. An analysis of the binding site and the areas surrounding it shows that the region in close proximity to the catalytic site contains multiple "ridges" and "grooves." Although the majority of the HDAC inhibitors are able to bind immediately outside the catalytic site, there are no computer-aided and medicinal chemistry studies that would systematically map the more distant grooves and ridges outside the catalytic site using the small molecules HDACi as probes and correlate them with the activity of the ligands. We propose to address this problem with the following specific aims: (1) Design and synthesize lead- and drug-like inhibitors of HDAC8 able to probe the area outside the catalytic site. To achieve this, use computer-aided drug design (CADD) and machine learning methods in concert with medicinal chemistry. The CADD will include de novo/rational drug design, virtual focused combinatorial library (vFCL) generation, in silico screening of vFCL; (2) assay candidate ligands for their HDAC8 enzyme activity inhibition; (3) identify the spatial structural motifs crucial for disruption of the protein-protein, e.g., HDAC8-histone, recognition and achieving the maximum inhibition of HDAC8. Potent HDAC8 inhibitors with low micromolar or nanomolar activity will be subjected for further optimization of ligand activity through iterative rounds of CADD, chemical synthesis, and biological tests for HDAC8 inhibitory activity.

An assay for HDAC8 activity was set up and validated. Fifty plus new HDAC ligands were designed, synthesized, and tested for HDAC8 inhibitory activity. The molecular dynamics simulation of several HDAC ligands suggests that the SBGs of the HDAC inhibitors can occupy different binding positions depending of the bulk and nature of the CAP groups. All ligands were docked to the binding site of HDAC8 and the binding modes of the ligands were compared with each other and with the binding modes predicted by the molecular dynamics simulations. Based on these studies, a new series of ligands targeting the areas important for activity and selectivity are currently designed and synthesized. The details of the studies and the activity of the most recent series of compounds will be presented.

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P49-21: DELIVERY OF ANTIVASCULAR DRUGS TO IRRADIATED BREAST TUMORS

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Temple University

Background and Objectives: The radiation-induced up-regulated expression of endothelial cell adhesion molecules provides a potential avenue for targeting drugs to irradiated tissue. Previously we and other investigators have shown the feasibility of this approach, in both irradiated normal tissue and irradiated tumors (*Pharm Res.* 22(7):1117-1120, 2005). E-selectin receptors are expressed on actively proliferating endothelium and are further upregulated in vasculature of irradiated tumors. Anti-E-selectin conjugated immunoliposomes can be used to target drugs (e.g., antivascular drugs) to solid tumors. Our goal is to evaluate the effects of preferentially targeting the antivascular drug combretastatin to irradiated mouse mammary tumors.

Methods: Combretastatin disodium phosphate (CA4P) was incorporated into liposomes with surfaces modified by the addition of a mouse monoclonal antibody to E-selectin, to create an immunoliposome (IL). This addition of anti-E-selectin allows the liposome to be preferentially targeted to E-selectin. Both single and fractionated dosing schemes were examined in this experiment. C3H mice bearing transplanted MCA-4 mouse mammary tumor were randomly assigned to one of the following treatment groups: Single Dose; untreated, systemic administration of free CA4P (15 mg/kg of CA4P), liposomes (L) alone (15 mg/kg of CA4P), IL alone (15 mg/kg of CA4P), irradiation (IR) alone (5 Gy), L + IR, IL + IR, and free CA4P + IR, Fractionated Doses; untreated, fractionated irradiation (Fr IR) 20 Gy total, multiple doses of L (15.0 mg/kg of CA4P), multiple doses of IL (15.0 mg/kg of CA4P), multiple systemic administration of free drug (81.0 mg/kg of CA4P), multi-L + Fr IR, multi-IL + Fr IR, and multi-free CA4P + Fr IR.

Results: Immunohistochemical staining indicated that E-selectin is upregulated in the vasculature of irradiated tumors 6, 24, and 48 hours post-irradiation. For single dose treatments, the group that received a single 5 Gy dose of irradiation plus a single dose of IL showed a significant delay in tumor growth compared to all other treatment groups. However, at the end of study, the growth delay of fractionated irradiation plus multiple doses of immunoliposomes was not significantly different from the other treatment groups that combined radiation and CA4P.

Conclusions: Targeting of antivascular drugs to irradiated tumors via ligand-bearing liposomes results in significant tumor growth delay. The use of pegylated liposomes with ligand attached to the distal end of PEG chains appears to be the most appropriate way to combine long-circulating properties, critical for delivery of antivascular drugs to tumors, and binding of liposomes to endothelium of tumor vasculature. We are currently investigating the biodistribution of immunoliposomes in irradiated tumors to determine mechanisms by which these drug carriers target tumor vasculature.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0269.

P49-22: MIGRASTATIN ANALOGUES AS POTENT INHIBITORS OF BREAST CANCER METASTASIS

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Metastasis is the multistep process wherein a primary tumor spreads from its initial site to secondary tissues/organs. Since breast tumor spreading is responsible for the majority of deaths of breast cancer patients, development of therapeutic agents that inhibit breast tumor metastasis is highly desirable. Migrastatin is a macrolide natural product first isolated from a cultured broth of *Streptomyces* and its structure features a 14-membered macrolactone ring. At high μM concentrations, the natural product inhibits the migration of several types of tumor cells in vitro but has no effect on the biosyntheses of DNA, RNA, and protein in these cells. We have shown that some synthetic migrastatin analogues are much more potent (by 3 orders of magnitude) than the migrastatin parent natural product at inhibiting breast tumor cell migration in vitro. In contrast, migration of normal human mammary gland epithelia cells and primary leukocytes was rather insensitive to these compounds. We have tested two synthetic migrastatin analogues, a core macroketone and a core macrolactam, on inhibiting lung metastasis of highly metastatic mammary carcinoma cells in a mouse model. These two readily synthesized compounds are potent inhibitors of mouse breast tumor metastasis reducing 91%–99% of tumor spreading to the lung. We have further shown that the cellular basis for this effect is the interference of the formation of lamellipodia that, in turn, inhibits migration of breast tumor cells. We have identified the protein target of these migrastatin analogues and this protein is highly expressed in invasive breast tumors. Therefore, one can foresee the promising potential these migrastatin analogues hold for treating breast cancers in combination with other approaches.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0362.

P49-23: DEVELOPMENT OF PHAGE PANNING METHODS FOR TUMOR-SPECIFIC LIGANDS WITH LASER CATAPULT MICRODISSECTION

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Background: Phage display technology has been widely used for developing tumor-targeting agents. Laser catapult microdissection (LCM) has proven to be an accurate method to select specific cells from histological sections.

Objective: To discover single-chain variable fragment antibodies (scFv) specific to tumor cells, an M13 phage-displayed scFv library was panned on LCM-captured tumor cells from a clinically removed human colon cancer tissue and screened by immunofluorescence stain on tumor tissues from the same patient.

Methods and Results: Two panning strategies were evaluated and optimized. The first strategy was to pan on patient tumor tissue mounted to LCM slides before LCM occurred. The poor panning output showed that phage did not tolerate conditions during LCM. The loss of phage viability was related to drying of the specimens, even when covered with anti-drying reagents, such as 5% glycerol buffer. The second strategy was to pan on the tumor cells from the patient tumor tissue after being isolated by LCM. The catapulted tumor cells were transferred to a filter unit that retained cells but allowed rinsing of unbound reagents, including unbound phage. Six commercially available filter units were evaluated and the one with the lowest nonspecific binding to phage was selected for the panning steps. The smallest number of cells (500) in which panning could be successfully accomplished was also determined. However, after 5 rounds of series panning on LCM-captured human tumor tissue, undesirable filter binders were selected rather than a binder that was specific to tumor tissue. These results showed that the background level in this system was still high when working on a small amount of tumor tissue. To minimize background binding, a micropipette was used to remove catapulted cells from the working filter unit to a new blank unit after panning was complete. This left behind nearly all background binding phage in the filter unit.

This strategy led to the selection, in only one round of panning, of individual scFv clones (5 out of 76 tested) specific to tumor cells of the patient's tumor tissue. Immunofluorescence stain on tumor tissues from the same patient showed that these clones had selective signals on tumor island cells while the scFv library only showed low nonspecific signals on tumor tissues.

Conclusion: We established a method of panning on small number of LCM-captured tumor specimens. This method can be used to any solid tumors including breast cancer.

We hope to develop this method to be able to rapidly identify phage-displayed scFvs that bind preferentially to subpopulations of tumor tissue, including tumor cells, stroma, and blood vessels. The quick identification of specific phage-displayed scFv antibodies to the tumor tissue of human patients will greatly enhance the therapy and diagnosis of tumor.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0237 and Vermont Cancer Center at the University of Vermont.

EXPERIMENTAL THERAPEUTICS II

Poster Session P50

P50-1: ENHANCING HORMONAL THERAPY FOR BREAST CANCER BY COMBINATION WITH VALPROIC ACID A WELL-KNOWN APPROVED PHARMACEUTICAL WITH LITTLE TOXICITY

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The beneficial effects of hormonal therapy for estrogen receptor (ER)-positive metastatic breast cancer are limited by the phenomenon of hormonal resistance. Similarly, hormonal therapy to prevent recurrence after successful surgery is not always effective. One attractive tactic to extend the magnitude and duration of response to hormonal therapy would be to combine it with another therapy with an independent target in the tumor cells. Unfortunately, the most familiar of such candidate therapies, cytotoxic agents used in chemotherapy, do not combine effectively with hormonal therapy, which appears to antagonize the beneficial actions of chemotherapy as observed both in clinical trials and in cell culture models. We have found that valproic acid (VPA), a pharmaceutical long used as an anticonvulsant and recently found to have antitumor action possibly because it is a histone deacetylase inhibitor, enhances the action of antiestrogens and aromatase inhibitors. Furthermore, valproic acid prevents the pro-proliferative effects of tamoxifen on uterine cells, suggesting that the combination of tamoxifen and valproic acid might be both more effective and less potentially harmful than tamoxifen monotherapy. VPA is currently dosed to reach a target concentration in patient serum. VPA treatment at the recommended concentrations enhances the actions of tamoxifen, raloxifene, or fulvestrant to prevent proliferation of estrogen receptor-positive breast cancer cells in culture. Thus, tamoxifen blocks cell cycling in these cells, and VPA both potentiates tamoxifen and makes it more efficient by cooperating with it to induce apoptosis. Our specific hypothesis is that VPA will enhance the effectiveness and duration of hormonal therapy of human breast cancer xenografts growing in mice and with few side effects. Thus, VPA would be an immediate candidate as an agent to enhance the effectiveness of hormonal therapy in humans. Specific Aims: (1) To examine the ability of VPA to enhance both (a) the initial response and (b) the duration of response to the antiestrogen tamoxifen or to estrogen ablation therapy of MCF-7 xenografts grown in ovariectomized nude mice and to study the effects of VPA on markers of cell cycling, apoptosis, and gene expression in the xenografts. (2) To examine the ability of VPA to enhance the extent of initial response and duration of response to the aromatase inhibitor letrozole of MCF-7/aromatase cells growing as xenografts under stimulation with adrenal androgen in ovex nude mice. We will present studies showing both the effect and potential mechanisms whereby VPA enhances hormonal therapy in cell culture and (if ready) some preliminary results of our studies in mice.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0501.

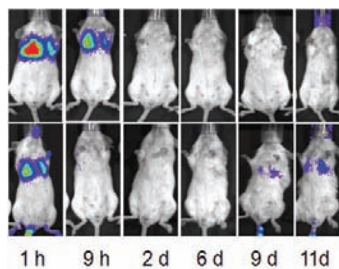
P50-2: TRAFFICKING THE FATE OF MESENCHYMAL STEM CELLS IN VIVO BY BIOLUMINESCENCE IMAGING

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Mesenchymal stem cells (MSCs) are considered to be a promising source of autologous stem cells in personalized cell-based therapies. The tumor homing property of MSCs provides an attractive opportunity for targeted transgene delivery into the site of tumor formation. In this study, we introduced a fusion reporter gene fLuc-eGFP and followed the MSC distribution and differentiation with in vivo bioluminescence imaging (BLI) and ex vivo GFP detection. Both subcutaneous breast cancer model and breast cancer lung metastasis model were established by subcutaneous and intravenous injection of 4T1-rLuc-mRFP cells, respectively.

The MSC-Fluc-eGFP cells (10^5 cells/mouse) were intravenously injected 4 days after 4T1-rLuc-mRFP i.v. injection or 1 week after s.c. inoculation of 4T1-rLuc-mRFP cells. The fate of MSC-Fluc-eGFP cells was monitored by fLuc BLI. The co-localization of MSC-Fluc-eGFP and 4T1-rLuc-mRFP cells was confirmed by ex vivo fluorescence microscope observation. The transfected MSCs were CD90⁺CD44⁺CD117⁺CD106⁺CD34⁺CD45⁺CD117⁺ and GFP⁺ and retained the ability to differentiate into osteoblast and adipocyte in vitro. When the MSCs were injected into normal mice, the fLuc signal in lung area decreased to background level 2 days p.i. and kept at background level until the end of experiment. In contrast, when the MSCs were injected 4 days p.i. of 4T1 cells, the fLuc signal in the lung area decreased to background level 2 days after injection and gradually went up from day 7 to day 14. In the



Tumor homing character of MSC:
top, normal Balb/C mouse; bottom,
mouse with 4T1 lung metastasis.

s.c. 4T1 tumor model, no fLuc signal was detected at tumor area until 6 days after MSC injection. Ex vivo fluorescence microscope observation confirmed the co-localization of GFP⁺ MSCs and RFP⁺ 4T1 cells in both s.c. tumor and lung metastasis. H&E staining of the lung tissue confirmed the presence of multiple tumor nodules in lung. Alizarin red S staining observed massive calcium deposit in lungs, suggesting osteoblast differentiation of injected MSCs in the lung metastasis. H&E staining of s.c. 4T1 tumor after MSC injection observed multiple unstained bubbles inside the solid tumor, suggesting the existence of potential lipid vacuoles, which was further confirmed by Oil red O staining. Anti-GFP staining further confirmed that GFP⁺ cells are located around the lipid vacuoles. In conclusion, both s.c. breast cancer and lung metastasis can attract MSCs. The tumor microenvironment has a profound effect on the differentiation pattern of MSCs after intravenous injection. Our future study will include the investigation of MSC differentiation mechanisms under different microenvironments and introduction of therapeutic gene for breast cancer therapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0374.

P50-3: DEVELOPMENT OF A NATURE-INSPIRED VECTOR FOR TARGETED BREAST CANCER GENE THERAPY

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Background: The target sites for nucleic acid molecules used in gene therapy are mostly in the cell nucleus. Therefore, for vectors to be maximally effective, they should condense pDNA to protect them from serum endonucleases, be recognized and internalized by target cells, promote escape from the endosomal compartment, and direct the nucleic acids toward the nucleus for transcription. A vector was engineered equipped with a DNA condensing motif obtained from adenovirus μ peptide to condense pDNA, an endosome-disrupting motif to promote escape of pDNA into cytosol, a nuclear localization signal to facilitate localization of pDNA in nucleoplasm, and a targeting peptide to specifically target human breast cancer cells.

Objective: The objective of this research is to develop a gene delivery system that can target breast cancer cells specifically and transfect them efficiently.

Methods:

Biosynthesis of the Targeted Vector: The nucleotide sequence encoding the vector was synthesized and cloned into a pET21b expression vector. The expression vector was transformed into competent *Escherichia coli* BL21DE3 pLysS cells and expressed. The expressed vector was purified using Ni-NTA column chromatography. The purity and expression was identified by SDS-PAGE and western blot analysis.

Particle Size Analysis: Various amounts of vector were complexed with 2 μ g pEGFP to form nanoparticles and characterized in terms of particle size using Malvern Particle/Zeta sizer.

Cell Transfection: ZR-75-1 breast cancer and MCF-10A normal human mammary cells were seeded in 12-well tissue culture plates at 70,000 cells per well. Cells were approximately 80% confluent at the time of transfection. pEGFP was mixed with various amounts of vector for complex formation. The complexes were added to the growth media supplemented with 10% serum. The expression of GFP and percent transfected cells was determined by an epifluorescent microscope.

Results: Vector Biosynthesis: The western blot analysis using anti-histag antibody confirmed the expression of the vector. The SDS-PAGE results demonstrated that the purity of the vector was >98%.

Particle Size Analysis: The results of the vector/pDNA complexation studies showed that the purified vector was able to condense pDNA into nanosize particles with 78 ± 8 nm size suitable for cellular uptake.

Transfection Studies: The results demonstrated that the vector/pEGFP complexes were able to transfect $45 \pm 8\%$ of the ZR-75-1 cells in the presence of serum. Approximately 5% of the MCF-10A cells were transfected, which could be due to the nonspecific uptake of the particles.

Conclusion: It was concluded that the proposed vector is able to condense pDNA efficiently into nanosize particles and transfect breast cancer cells efficiently with minimal nonspecific uptake by normal mammary cells. Additional optimization studies are currently under investigation.

Impact: Once the concept is proven, additional carriers can be custom-designed, programmed, and developed to deliver therapeutic genes to various subpopulations of target breast cancer cells. In this research, a 13aa breast cancer-targeting peptide has been utilized as a targeting motif, but in future work other targeting motifs could be utilized including antibodies, growth factors, or aptamers specific to receptors expressed on the surface of breast cancer cells.

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P50-4: TUMOR HOMING PEPTIDE DIRECTED ONCOLYTIC ADENOVIRUS TARGETING BREAST TUMOR LYMPHATIC AND ABERRANT TGF β SIGNALING: A NOVEL APPROACH FOR BREAST CANCER THERAPY

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Background: The goal of this project is to develop a gene therapy approach to treat breast cancer. We have chosen to develop oncolytic adenoviral (d101/07 based) based therapy as d101/07 can replicate in all breast cancer cells. We tested the hypothesis if LyP-1 (a cyclic peptide that binds with breast tumor lymphatic) directed adenovirus AdLyP1.sTBRFc, would have additional tumor specificity. In addition, we have targeted growth factor-beta (TGF β) pathway because high levels of TGF β and SMAD signaling are known to contribute to the tumor progression.

Objectives: To create AdLyP1.sTBRFc and Ad.sTBRFc and examine if these viral vectors (1) will be oncolytic to the tumor cells and produce sTGF β RIIFc; (2) sTGF β RIIFc will abolish the effects of TGF β ; (3) the administration of viruses either intravenously into the MDA-MB-231 tumor bearing mice or into the human xenografts tumors in nude mice will result in the inhibition of tumor growth.

Methods: Ad.sTBRFc and AdLyP1.sTBRFc were generated by homologous recombination. Breast tumor cells were infected with viral vectors and protein expression measured by western blots and ELISA. Viral toxicity was examined by cell survival assays and plaque forming assays. To study the effect of sTGF β RIIFc on TGF β 1 signaling, the proteins were mixed and SMAD-3 phosphorylation by western blots; IL-11 production by ELISA; and osteoclast formation by counting TRAP positive multinucleated cells were examined. To examine anti-tumor responses, MDA-MB-231 tumors were established in nude mice, viral vectors injected into the tumors or intravenously, and tumors sizes and animal survivals were examined once a week.

Results: AdLyP1.sTBRFc and Ad.sTBRFc were produced by homologous recombination. Infection of breast cancer cells with Ad.sTBRFc and AdLyP1.sTBRFc produced high levels of sTGF β RIIFc that was released into the extracellular medium (greater than 2 μ g/ml in the medium). There was significant viral replication (up to 15,400-fold increase in viral burst size) in the breast tumor cells. Purified sTGF β RIIFc was shown to bind with TGF β -1, causing inhibition of TGF β -dependent SMAD-2 phosphorylation, IL-11 production, and osteoclast formation. Intratumoral or systemic administration of Ad.sTBRFc or AdLyP1.sTBRFc in the pre-established MDA-MB-231 human breast xenografts in nude mice tumors induced significant anti-tumor responses (p values <0.005). High levels of sTGF β RIIFc were detected in the blood, indicating that it is feasible to achieve high viral replication and gene expression in vivo.

Conclusion and Impact on Breast Cancer Research and Treatment: Data presented here shows that it is feasible to create Ad.sTBRFc and AdLyP1.sTBRFc, achieve tumor specific viral replication, and simultaneously produce sTGF β RIIFc that inhibits of TGF β signaling, and anti-tumor responses. These results suggest that Ad.sTBRFc AdLyP1.sTBRFc can be developed as anti-tumor agents for the treatment of breast cancer.

Publications:

1. Ayer et al. 2005. *Cancer Biol Ther.* 4:261-266.
2. Wang et al. 2006. *Mol Cancer Ther.* 5:367-373.
3. Seth et al. 2006. *Hum Gene Ther.* 17:1152-1160.
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P50-5: DEVELOPMENT OF TARGETED BREAST CANCER THERAPY USING BI-FUNCTIONAL APTAMERS

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The specific goal of this research is to rationally design multifunctional RNA aptamers that will reduce or destroy proteins implicated in breast tumor development or progression. To accomplish this goal, we developed a model system to prove this novel concept and generated RNA aptamers for a specific intracellular target.

First, high affinity aptamers were selected from a library using a target protein via SELEX. The resulting sequences were organized into two different aptamer groups based on sequence homology. The important regions of the two aptamers were mapped to identify the smallest possible active structure. The smallest aptamer developed from the Group-1 sequence is 40 nucleotides long and has a Kd ~ 80 nM. Next, we created a bi-functional aptamer using the Group 1 aptamer and a pre-existing aptamer

with affinity for green fluorescent protein (GFP) and tested for binding to each protein. The bi-functional aptamer was also tested in a cell-based assay. In this assay the aptamer was first incubated with target and GFP proteins and then incubated with cells. Effects were observed by fluorescence microscopy and positive signals were detected in the cellular cytoplasm. These results provide a critical test of the first step in our hypothesis that bi-functional aptamers can be used to reduce the level of an extracellular protein.

In a majority of all breast cancer, estrogen receptor (ER) is overexpressed and estrogen is recognized as a cancer cell mitogen. Therefore, we chose human ER α as our first intracellular target protein. We used full length ER α expressed from a baculovirus construct in insect cells to perform an in vitro selection experiment and identified three distinct aptamers. In the relatively small sample we sequenced, one of these aptamer sequences occurred 9 times and the other two each occurred once. We have measured their affinity to ER using both filter binding and electrophoretic mobility assay. The complex of ER with two of these aptamers have dissociation constant in the 10 nM range, the third one has a Kd of 30 nM. We are in the process of pinpointing the aptamer binding site on the protein. Previous works indicate that a DNA binding protein is likely to select RNA aptamers for the DNA binding site. This type of aptamer may prevent binding of ER to its responsive element and may have potential as a pharmaceutical. They will also be useful as components of bi-functional aptamers when an intracellular system of induced destruction is developed in the future.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0599.

P50-6: CREATING ONCOLYTIC ADENOVIRUS TARGETING TUMOR LYMPHATICS

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University of Chicago

Tumor metastasis is a major cause of death among cancer patients. The lymphatic vessels are an important route for the spread of solid tumors such as in breast cancer. It is known that VEGF family members, VEGFC and VEGFD and their receptor VEGFR3, play significant roles in the proliferation of new lymphatic vessels, which may induce tumor lymphangiogenesis and direct metastasis to the lymphatic vessels. Destroying tumor lymphatics might be an effective way in controlling lymphatic spread of breast tumors. In the proof-of-principle studies, we created oncolytic adenoviruses by incorporating tumor cell and tumor lymphatics targeting peptide (LyP-1) into the adenovirus fiber protein and characterized their improved efficiency of delivery into tumor cells and endothelial cells. Further, VEGFC isoforms were also identified for the study of their roles in cell signaling and tumor growth.

We constructed adenoviruses containing LyP-1 peptide inserted into the fiber H1 loop by homologous recombination. The trimerization of fiber protein was unaffected by incorporation of short tumor homing peptide in the fiber H1 loop. The assembly of the modified virus was normal compared with the unmodified virus. The modified virus showed increased transduction efficiency in MDA-MB-435 breast cancer cells. The increased transduction efficiency was also accompanied by enhanced cytotoxicity. In a luciferase assay, the modified virus exhibited improved delivery into human dermal lymphatic endothelial cells and mouse kidney microvascular endothelial cells. These results indicated that the targeting peptide inserted into the fiber H1 loop was functional in the viral context. In our work with VEGFC, we discovered three short isoforms of VEGFC through the cloning of the VEGFC gene. These isoforms have peptide sequences of 184, 129, and 62 amino acid residues, respectively, resulting from products of alternative RNA splicing. The connection between exons and introns is in agreement with consensus sites of RNA splicing. The C-terminal part of VEGFC is missing in these isoforms due to frameshifts of the sequences. The conditioned media from HEK-293 cells transfected with different forms of VEGFC stimulated phosphorylation of tyrosine and serine residues in a different pattern in immortalized podocyte cells, indicating different downstream signaling pathways. MDA-MB-435 cells stably transfected with one isoform displayed a reduced growth phenotype compared with control cells. These isoforms will be placed in the adenovirus to study their effects on the growth of tumor cells and lymphatic endothelial cells and their roles in lymphangiogenesis.

Given the role of tumor-associated lymphatics in lymph node metastasis, the development of oncolytic adenovirus targeting tumor lymphatics will provide us new tools to combat tumor growth and metastatic spread. Future work will be concentrated on the optimization of insertion of the peptide in the viral context for maximal binding efficiency and specificity for tumor lymphatic vessels. Further investigation into the functions of VEGFC isoforms will also help us to fully understand lymphangiogenesis in both normal and pathological conditions in order to generate novel delivery vehicles to block VEGFC-induced lymphangiogenesis in the tumor microenvironment.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0467 and National Institutes of Health.

P50-7: RECOMBINANT ADENOVIRUS EXPRESSING CONSTITUTIVELY ACTIVATED INTERFERON REGULATORY FACTOR 3 SUPPRESSES TUMOR GROWTH IN A HUMAN BREAST CANCER MOUSE MODEL

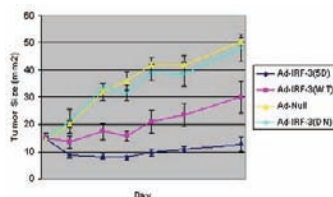
John H. Yim,¹ Ki-Young Sung,² Michael T. Stang,¹ Baoguo Ren,¹ Michaele J. Armstrong,¹ and Ye Liu¹

¹University of Pittsburgh and ²Catholic University of America

Introduction: Interferon Regulatory Factor-3 (IRF-3) is important in the signaling of the innate immune response. In response to an insult, IRF-3 becomes activated and translocates to the nucleus, which results in transcriptional activation of genes against the insult. We utilize a recombinant adenovirus (rAd) that expresses a mutated constitutively activated IRF-3 that results in immediate and persistent transcriptional upregulation of IRF-3-regulated genes (Ad-IRF-3[5D]). We hypothesize that expression of these genes results in tumor suppression of breast cancer cells.

Methods: rAds encoding wild-type IRF-3 (Ad-IRF-3[WT]), Ad-IRF-3(5D), and dominant-negative IRF-3 control (Ad-IRF-3[DN]) were propagated. Infected cells were immunoblotted for IRF-3, and ELISA was performed for human IFN- β . MDA468 cells were injected in the mammary line of SCID/Beige mice. Established tumors of mice (n=5 per group) were injected with adenovirus or empty vector adenovirus control (Ad-Null) three times in one week. Measurements are given as mean tumor size (width x length) \pm SEM.

Results: Immunoblotting for IRF-3 confirmed large production of each IRF-3, and functional expression of the Ad-IRF-3(5D) was confirmed by ELISA of marked secretion of IFN- β into the media by infected cells. Established MDA468 tumors injected intratumorally with rAd show growth suppression Ad-IRF-3(5D)>Ad-IRF-3(WT)>controls (Figure).



Conclusions: IRF-3 may mediate the innate immune response against breast cancer. Intratumoral injection of Ad-IRF-3(5D) results in marked tumor suppression while Ad-IRF-3(WT) shows less but significant suppression versus control injected tumors. Tumor suppression may result from autocrine and paracrine effects of IFN- β . Manipulation of the innate immune response, such as with gene therapy with IRF-3, may result in a clinical benefit against human breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0459.

P50-8: SECRETION OF SOLUBLE ERYTHROPOIETIN RECEPTOR BY GENETICALLY ENGINEERED BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS

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Cancer cells can dispense signals that command the production of new blood vessels to feed the tumor as it grows. One such angiogenic signal is erythropoietin (Epo). Breast cancer may progress to a likely incurable state when the extent of blood supply to the tumor permits it to grow and spread to vital organs, that is to metastasize. Obstructing the recruitment of new blood vessels to the tumor will hinder cancer progression. One such anti-angiogenic approach may involve cell and gene therapy. We hypothesize that normal bone marrow-derived mesenchymal stromal cells (MSCs) can be harvested from patients and genetically engineered to release a therapeutic protein, specifically the soluble Epo receptor (sEpoR), an antagonist to cancer-associated Epo. The MSCs would be gene modified in the laboratory and then returned to the patient as a safe and removable implant continuously releasing sEpoR that should interfere with cancer neovascularization and progression. We therefore set out to test this strategy in mouse models of breast cancer. We generated several vector constructs that we analyzed and tested on cell lines prior to gene modifying primary murine MSCs. The best transgene expression was achieved utilizing a mammalian expression vector in which we introduced the cDNA for human sEpoR. This vector also contains a drug resistance gene that allowed us to drug select and expand stably transfected murine MSCs expressing the sEpoR. With our resulting sEpoR gene-modified MSCs, we were able to detect the sEpoR by western blot analysis using the cell supernatant and reconfirmed efficient gene transfer through the success of other western blots with different antibodies. Our most optimal vector construct also comprises a polyhistidine tag at the 3' end so as to allow protein purification. Current studies are establishing the presence and potency of the ligand-receptor interaction between human Epo and the sEpoR secreted by the sEpoR gene-modified MSCs.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0538.

P50-9: TARGETING FOXP3 EXPRESSION IN Tregulatory CELLS USING PHOSPHORODIMIDATE MORPHOLINO OLIGOMERS: POTENTIAL TO DEVELOP A NOVEL ADJUVANT FOR BREAST CANCER IMMUNOTHERAPY

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A successful anticancer immunotherapy requires not only potent T cell activation but also strong interference with mechanisms of immune tolerance mediated in part by CD4+CD25+ regulatory T cells (T_{reg}). T_{reg} cells seem to negatively regulate antitumor immunity by dictating the magnitude of immune responses to tumor-associated self-antigens stimulated by targeted immunotherapies that include *HER2* oncogene, an attractive target for breast cancer immunotherapy. We hypothesize that targeted depletion of CD4+/CD25 regulatory T cells using a genomics-based strategy represents a novel adjuvant for immunotherapeutic strategies in breast cancer. Expression of scurf transcriptional regulator encoded by the member of the forkhead winged helix protein family (FOXP3) have been identified to be critical for maintaining function of T_{reg} cells and sufficient to induce a suppressor phenotype in CD4+CD25- T cells. Therapeutics that destroy or inhibit the function of the T_{reg} compartment should allow optimization of cancer immunotherapies and a resultant induction in efficacious antitumor responses. In this study, we have developed antisense phosphorodiamidate Morpholino-based oligomers (PMO), a genomics-based strategy with a unique chemistry and mechanisms of action for targeted drug development with high specificity and low toxicity. A plasmid-based screening system has been established to screen various PMO sequences to reveal optimal and specific antisense sequences to target *FOXP3* gene. In addition, studies are being carried out to understand the kinetics of PMO uptake in cancer cells and in immune cells and correlation with inhibition of target protein translation. Studies with neutral PMO compounds show high uptake in primary cells compared to established cancer cell lines. Differential PMO uptake was observed in immune cells with macrophages and monocytes showing highest uptake compared to T and B cell. We have now generated unique peptide-tagged PMO compounds including those targeting FOXP3 that can be efficiently delivered into immune cells including Tregulatory cells isolated from human peripheral blood mononucleocytes (PBMC) from leukopheresis. PMO agents in general have shown excellent safety in clinical trials that strengthens the feasibility of extending these studies to the clinic in the near future potentially in combination with the widely used *HER2*-based immunotherapies in breast cancer.

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P50-10: RETROTRANSPON-MEDIATED CANCER GENE THERAPY

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Suicide gene therapy for cancer would be more effective if techniques for specifically targeting tumor cells improved. At present, delivery of suicide genes is imprecise, and normal cells and tissue may also succumb to treatment. This research explores the use of the human LINE-1 (L1) retrotransposon as a vector for delivering suicide genes into solid tumors. Human L1s are non-viral mobile DNA elements that duplicate themselves in the genome by a "copy and paste" mechanism using an RNA intermediate. It was previously shown that it is possible to insert a reporter cassette into the 3' untranslated region of a retrotransposition-competent L1. When this construct is placed in an episome and introduced into cells in culture, the reporter gene is expressed only following retrotransposition of the L1 into genomic DNA. Furthermore, it has been observed that L1s are active in many tumor cell lines, but suppressed in primary cells. This suggests that the L1 might be used as a vector to specifically target tumor cells, while minimizing damage to surrounding tissues.

We provide evidence that the L1 is more active in tumor cell lines, including breast cancer cells, than related primary lines. Currently, we are testing L1 vectors containing luciferase and green fluorescent protein reporter cassettes for their ability to retrotranspose in tumor xenografts in nude mice, and are working to improve retrotransposition efficiency. There are two strategies for introducing the L1-reporter cassette into cells following tumor injection: in a plasmid vector using polyethylenimine (PEI) gene transfer reagent, or by infection with a "gutted" adenovirus-L1 chimeric virus (Kubo et al., 2006, *PNAS* 103, 8036). We intend to assay L1-delivered suicide gene cassettes developed from a modified caspase-3 gene (Srinivasula et al., 1998, *J. Biol. Chem.* 273, 10107) and the yeast cytosine deaminase gene for their ability to retard growth of xenografts.

Retrotransposon-mediated intratumoral gene transfer could potentially be a novel therapy allowing long-term gene expression by integration. The tumor specificity of L1 retrotransposition should limit damage for normal tissues and increase the efficiency of “suicide” gene therapies for some solid tumors.

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P50-11: EARLY BLOOD-BASED DETECTION AND MONITORING OF BREAST CANCER WITH A DUAL-REPORTER UNDER THE CONTROL OF A CANCER-SPECIFIC PROMOTER

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Improvements are needed for detection of non-palpable breast cancer at an earlier stage. Current imaging methods lack sensitivity to detect sub-millimeter size tumors and there is currently no blood-based screening approach that can be applied for this purpose. We propose that early diagnostic and monitoring of breast cancer can be achieved with a dual genetic system that includes a human secreted embryonic alkaline phosphatase (SEAP) reporter for blood-based screening and a fluorescent reporter (mCherry) for imaging, both under the control of the cancer-specific Id1 promoter. Id1 is a member of the inhibition of differentiation gene family that encodes helix-loop-helix proteins that inhibit transcription by forming inactive heterodimers with basic helix-loop-helix (bHLH) proteins. Id1 is upregulated in association with breast tumor angiogenesis. SEAP is not expressed in adults, expression will not induce an immune response since it is of human origin, and it can be detected specifically in blood with high sensitivity. The application of the image-based reporter mCherry will allow for optical imaging and localization of breast tumor nodules with high sensitivity and decreased tissue attenuation. Our goal is to develop the cancer-specific diagnostic Ad vector Ad5-Id1-SEAP-Id1-mCherry with the Id1 promoter, controlling expression of each reporter to allow for maximal expression. Analysis of an Ad5 vector encoding firefly luciferase under control of Id1 showed high levels of expression in 2LMP (derived from MDA-MB-231) and T47d breast cancer cells. The Ad5-Id1-SEAP-Id1-mCherry vector production includes combination of the two cassettes in pShuttle and subsequent bacterial-based homologous recombination using pAdeasy to obtain the Ad vector. The dual reporter construct is thereby incorporated into the E3 region of replication-competent Ad5 vectors to improve imaging sensitivity with a blood-based screening system. Inflammation induced by the Ad vector will be addressed with the use of mesenchymal stem cells as a cell-based delivery of the vector. Success of the strategy proposed in this project will lead to an accurate and widely applicable method for early detection and monitoring of breast cancer.

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P50-12: DEVELOPMENT OF ADENOVIRAL LIGAND LIBRARIES FOR VECTOR TARGETING

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Adenoviral (Ad) vectors are one of the most robust gene delivery and oncolytic viruses for cancer therapy. In this application Ads have utility in delivering cytotoxic or immunostimulatory genes and their products to breast cancer cells to aid in debulking tumors or stimulating an effective immune response against primary and metastatic tumor sites. Adenoviruses are also being developed as oncolytic viruses to selectively replicate and kill cancer cells while sparing normal cells. While Ad shows utility in both applications, all natural Ads bind to promiscuous cell surface receptors. As such Ads frequently transduce or infect non-tumor sites (e.g., liver, spleen, and lung) much more effectively than breast cancer cells. In addition, many tumor cells downregulate the primary receptors for Ads rendering the cells relatively refractory to transduction or infection for gene or virotherapy. This lack of specificity translates into reduced therapy by “missing” the target. Because much of the vector is wasted on non-target tissues, one needs to deliver substantially larger doses of the vector to achieve sufficient gene delivery. This lack of specificity and delivery of large amounts of vector also increases the danger to patients since the genes are delivered into non-target tissues. In this project, we have engineered adenoviruses that display random peptide ligands and are testing the utility of this approach to make relatively large peptide libraries to select retargeted Ads against human breast cancer.

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P50-13: SYSTEMIC OSTEOPROTEGERIN GENE THERAPY RESTORES TUMOR-INDUCED BONE LOSS IN A THERAPEUTIC MODEL OF BREAST CANCER BONE METASTASIS

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Enhanced production of receptor activator of nuclear factor- κ B ligand (RANKL) and its binding to RANK on the osteoclasts have been associated with osteolysis in breast cancer bone metastasis. Osteoprotegerin (OPG) is a decoy receptor for RANKL and prevents RANKL-RANK interaction. The present study determined the effects of sustained expression of OPG using a recombinant adeno-associated virus vector (rAAV) in a therapy model of osteolytic breast cancer in mouse. Bone metastatic breast cancer was established by intra-cardiac injection of the human breast cancer cell line MDA-MB-435, constitutively expressing the firefly luciferase. Upon confirmation of skeletal metastases, mice were given a one-time intramuscular injection of rAAV encoding OPG.Fc or GFP. Mice were sacrificed 1 month later and the effects of therapy on tumor growth and bone remodeling were evaluated. Bioluminescence imaging showed significant reduction of tumor growth in bone in OPG.Fc treated mice when compared to GFP treated mice. Three-dimensional micro-CT analysis and histomorphometry of the tibia indicated significant protection of trabecular and cortical bones from tumor-induced bone loss following OPG.Fc therapy. Despite a significant prevention of the bone loss and tumor growth, OPG.Fc therapy failed to provide long-term survival, probably because of tumor growth in extra-osseous tissues. OPG.Fc treated mice developed more bone compared to age-matched normal mice indicating a requirement for regulated transgene expression. Results of this study indicate the potential of rAAV-OPG therapy for decreasing select lesions in osseous tissues for reducing morbidity and mortality in breast cancer patients with osteolytic bone damage.

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P50-14: A NOVEL PEPTIDE DISCOVERED BY INTRACELLULAR BACTERIOPHAGE DISPLAY MEDIATES NUCLEAR TARGETING

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Plasmid localization to the cell nucleus is a low probability event that contributes to inefficient transgene expression following nonviral gene delivery. Previous studies using a bacteriophage (phage) display approach yielded a panel of 7-mer peptides associated with nuclei of MCF7 human mammary epithelial cells. The phage clone with the highest frequency of nuclear recovery displayed the QPSPSPT peptide. The nuclear targeting capability of QPSPSPT indicates its potential as a novel ligand for modulation of transgene expression by nonviral gene delivery methods.

Nuclear localization of QPSPSPT was confirmed by image analysis of in vitro cell cultures exposed to fluorescently-labeled M13 phage displaying the heptapeptide. Control wild-type M13 phage and phage displaying the QPSPSPT peptide were labeled using an Alexa 488 fluorochrome. Purified fluorescent phage was incubated with MCF7 cells under standard culture conditions for 24 hours. Fluorescence microscopy revealed that 12.2% of the MCF7 cell nuclei were associated with labeled M13 phage displaying the QPSPSPT peptide, but wild-type M13 phage failed to localize to the cell nucleus.

Quantitative analysis of confocal fluorescence micrographs showed intracellular and intranuclear phage localization after 24 hours. The number of phage per MCF7 nucleus was 3.3-fold greater for samples displaying the QPSPSPT peptide relative to the wild type M13. Increased nuclear localization was mediated by heightened uptake of QPSPSPT-phage per cell (1.7-fold) and intracellular translocation of QPSPSPT-phage to the nucleus (1.9-fold) as compared to the wild-type M13 phage. The equivalent spherical diameter of a M13 phage is 40 nm, suggesting that the QPSPSPT heptapeptide has the capacity to mediate cellular entry and nuclear localization of payloads consistent in size with pDNA or formulated nonviral gene delivery vehicles.

Nuclear proteins associated with QPSPSPT were identified from MCF7 cell lysate using the peptide immobilized for affinity chromatography. Cell lysate fractions eluted by salt gradient from the immobilized QPSPSPT were isolated, separated by SDS-PAGE gel chromatography, digested by trypsin proteolysis, and assessed by high capacity ion trap mass spectroscopy (MS). MS peptide “fingerprints” were mapped on to the MSDB human protein database to identify cellular proteins associated with the QPSPSPT peptide. Neglecting band 1 (confirmed to be keratin-1), eight of the nine remaining bands (89%) retained by the QPSPSPT peptide were associated with nuclear proteins. For three of the nine bands (33%), the highest probability protein match was associated with the nucleus. At least 50% of the high probability proteins

are nuclear localized for another four of the nine bands (44%). Nuclear proteins with a high probability of QPSPSPT association include ribonucleoproteins, nucleophosmin, high mobility group box proteins, and histones.

Current work includes the conjugation of a pDNA encoding GFP with the QPSPSPT peptide to create a nuclear-targeted pDNA. This construct will be quantitatively assessed for nuclear delivery and transgene expression in MCF7 cells following formulation with a cationic lipid delivery vehicle. The capacity for QPSPSPT to enhance nuclear delivery and transgene expression will be assessed in comparison with control constructs lacking the heptapeptide.

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P50-15: ENHANCEMENT OF Bik ANTITUMOR EFFECT BY Bik MUTANTS

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Bik was initially identified as a BH3-domain-only protein that interacts with E1B 19K. Although systemically administered wild-type Bik significantly inhibited tumor growth and metastasis in an orthotopic nude mouse model, the proapoptotic potency of Bik can be modulated by posttranslational phosphorylation. Here, we found that Bik mutants, in which threonine 33 and/or serine 35 were changed to aspartic acid to mimic the phosphorylation at these two residues, enhanced their binding affinity with the antiapoptotic proteins Bcl-XL and Bcl-2 and were more potent than wild-type Bik in inducing apoptosis and inhibiting cell proliferation in various human cancer cells. Bik mutants also suppressed tumorigenicity and tumor-taking rate in a mouse *ex vivo* model. Moreover, Bik mutant-liposome complexes inhibited tumor growth and prolonged life span more effectively than the wild-type Bik-liposome complex in an *in vivo* orthotopic animal model. Finally, Bik mutant was driven under the breast cancer specific enhanced minimal Topoisomerase II composite promoter CT90, and it selectively killed breast cancer cells *in vitro* and suppressed mammary tumor development in an animal model. Expression of Bik mutant was detectable in the tumors but not in the normal organs. Our results provide an effective systemic breast cancer-targeting gene therapy.

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P50-16: NEW CONDITIONALLY REPLICATING ADENOVIRUS VECTORS FOR BREAST CANCER THERAPY

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Background and Objectives: Breast cancer is the most common form of cancer in the United States, and the second leading cause of cancer deaths among women. As a result, there is a pressing need to develop new treatments. Oncolytic, conditionally replicating adenoviruses (CRADs) represent one such approach. While initial clinical studies of oncolytic CRADs have shown that these vectors are generally safe and well tolerated, additional improvements are needed. Our objective to develop new CRADs containing mutant DNA polymerases with a high functional dNTP requirement; we hypothesize that these vectors will replicate selectively in tumor cells.

Brief Methodologies: Standard mutagenesis techniques were used to construct adenovirus type 5 vectors with selected mutations in the viral polymerase gene. The replication characteristics of these vectors were then assessed in cultured cells.

Results to Date: Mutations were made to key adenoviral polymerase motifs that are expected to interact with the dNTP substrate. Replication-competent virus molecular clones that contain these engineered mutations were constructed, and their replicative properties were evaluated in tumor-derived cell lines and in cells that were supplemented with exogenous deoxynucleosides (as a way to elevate intracellular dNTP levels). Out of an initial panel of 7 polymerase mutant virus constructs, one was found to be replication-competent (I664V). Transgene (GFP) expression from this vector was enhanced when cells were supplemented with exogenous deoxynucleosides. This suggests that the presence of additional dNTPs within the cellular environment either enhances viral replication or increases transgene expression at a transcriptional level.

Conclusions: Non-conservative mutagenesis of several selected polymerase motifs appears to compromise virus replication, while a conservative mutation within a conserved IxGG motif (I664V) was tolerated and gave rise to replication-competent virus. We are presently testing additional mutants, with a view to identifying viruses that replicate selectively in high dNTP environments, such as those found in breast cancer cells. It is expected that these new adenovirus vectors will provide an important tool for breast cancer treatment.

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P50-17: DISPERSIVE PHENOTYPE OF INSOLUBLE γ -TUBULIN IN METASTATIC BREAST TUMOR CELL LINES

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γ -tubulin is the major protein involved in microtubule nucleation in mammalian cells. In normal interphase human cells, γ -tubulin is thought to be found exclusively at the centrosome where, as part of the γ -tubulin ring complex (γ -TuRC), microtubule nucleation initiates. Microtubule disrupting agents such as paclitaxel have been used in cancer research to treat primary breast tumors. However, there is little evaluation of the effects of these drugs on metastasis. In this study, we sought to examine the role of γ -TuRC proteins in metastatic breast cancer to begin to elucidate the role of microtubules after intravasation into the circulatory system. Previous studies have reported that insoluble γ -tubulin may also exist outside of the centrosome, but the reasons for this remain unclear. Our current studies indicate that particular γ -TuRC proteins differ in their phenotypic localization among a panel of normal and tumorigenic human breast cell lines of increasing metastatic potential, when gauged by indirect immunofluorescence. Image analysis of fluorescence images revealed at least a fourfold increase of insoluble γ -tubulin expression in highly invasive breast cancer cell lines over a nontumorigenic and noninvasive cell lines. Furthermore, other γ -TuRC proteins, pericentrin and ninein, were also shown to be differentially expressed by indirect immunofluorescence. However, total levels of protein expression for γ -tubulin, pericentrin, and ninein remained at similar levels for all cell lines by SDS-PAGE analysis. Insoluble γ -tubulin localized outside the centrosome reinforces the evidence of microtubule nucleation in the cytosol, particularly along pre-existing microtubules. The possibility of microtubule nucleation along pre-existing microtubules has major implications on current breast cancer therapies that target microtubule stabilization, such as paclitaxel, which inhibits cell cycle progression at mitosis by disrupting microtubule growth of the mitotic spindle. However, drugs that inhibit primary tumor growth are often ineffective against disseminated metastatic tumor cells that often remain dormant for years before progression into a secondary tumor. Thus, drugs like paclitaxel may be ineffective against metastatic cells and potentially promote counterproductive microtubule growth in tumor cells with cytoplasmic γ -tubulin. Further investigation of the role of γ -TuRC proteins could reveal more effective ways to target the cytoskeleton of metastatic tumor cells for long-term treatments.

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P50-18: PHOTODYNAMIC MOLECULAR BEACONS AS SMART THERAPEUTICS TRIGGERED BY BREAST CANCER-ASSOCIATED PROTEASE AND mRNA

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Molecular beacons are target-activatable probes that use the fluorescent resonance energy transfer principle to control their fluorescence emission in response to specific biological stimuli. They are useful tools for cancer imaging because of their potential for improving imaging specificity (activation through tumor-specific enzymes or mRNAs) and sensitivity (signal amplification from non-fluorescent to highly fluorescent) as well as their ability to interrogate a wide range of molecular abnormalities. We have introduced the first therapeutic beacon family, called "photodynamic molecular beacons (PMB)." The PMB usually comprises a disease-specific linker (e.g., peptides or oligonucleotides), a photosensitizer and a quencher. This configuration allows the photosensitizer's phototoxicity to be silenced until the specific linker-target interactions (e.g., protease-mediated linker cleavage or nucleic acid hybridization-induced linker opening). Thus, the beacons can achieve a very high level of photodynamic therapy (PDT) treatment selectivity by destroying only the targeted cancer cells, while leaving non-targeted (normal) cells unharmed.

For example, we reported the first full implementation of this concept by synthesizing a breast cancer-associated matrix metalloproteinase-7 (MMP7)-triggered PMB and achieving not only MMP7-triggered production of singlet oxygen (the main cytotoxic species responsible for PDT) in solution but also MMP7-mediated photodynamic cytotoxicity in cancer cells. Preliminary *in vivo* studies also reveal the MMP7-activated PDT efficacy of this PMB. Moreover, we also successfully applied the PMB concept to develop the first PMB triggered by breast cancer-associated *c-raf-1* mRNA. When incubated with *c-raf-1* expressing MDA-MB-231 cancer cells, this mRNA-triggered PMB displayed remarkably efficient cellular uptake and subsequently effective PDT activation in these targeted cells. These studies validate the core principle of the PMB concept that the selective PDT-induced cell death can be achieved by exerting precise control of the photosensitizer's ability to produce singlet oxygen by responding to specific cancer-associated biomarkers. Thus, the PDT selectivity will in principle depend on how selective a biomarker is to cancer cells and how selective the interaction of PMB is to this biomarker.

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IMMUNE-BASED THERAPIES II

Poster Session P51

P51-1: ACTIVE IMMUNIZATION USING A PEPTIDE MIMIC OF A CARBOHYDRATE TUMOR ANTIGEN

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The tumor-associated carbohydrate antigen Thomsen-Friedenreich Antigen (TF-Ag) shows surface expression on many tumor cells types, including about 90% of breast tumor cells. This aberrant expression contributes to cancer cell adhesion and metastasis to sites containing TF-Ag-binding lectins. A specific IgG₃ monoclonal antibody developed to TF-Ag (JAA-F11) impedes TF-Ag binding to vascular endothelium in vitro, blocking a primary metastatic step and providing a survival advantage in a mouse breast tumor model after JAA-F11 passive immunization. Guided by these passive transfer results, vaccines that generate antibodies towards TF-Ag could be clinically valuable to breast cancer patients. Vaccinations with carbohydrate tumor-associated antigens conjugated with protein carriers have generated low immune responses in past trials. However, research shows that peptide mimics of carbohydrate antigens generate antibody to the saccharide antigens and form a memory response after peptide mimic immunizations. Further, T cells primed by peptide mimics can react with carbohydrate antigen-expressing cells. Therefore, we hypothesize that vaccinations with unique TF-Ag peptide mimics will generate immune responses to TF-Ag epitopes on tumor cells, which would be clinically applicable for active immunotherapy of breast and other cancers.

Peptide mimics of TF-Ag, selected through biopanning of a phage display library using the JAA-F11 antibody, were analyzed in vitro to confirm specific TF-Ag peptide mimicry by ELISA and immunoblotting experiments. In vitro model systems of cell adhesion demonstrated that the peptide mimics, like JAA-F11 antibody to TF-Ag, blocked rolling and stable adhesion of breast cancer cells to vascular endothelium. In vivo, multiple antigenic peptides were used in mouse immunizations, and sera analysis by ELISA showed the production of TF-Ag-reactive antibody. Statistically significant antibody levels reactive to TF-Ag were produced in several peptide mimic-immunized groups, including IgG antibodies, indicating the involvement of T cells. This result shows that these peptides can functionally mimic TF-Ag and generate potentially protective antibody with correct specificity. Additional work showed that MHC molecules are predicted to be able to present the peptides in vivo and that no known human proteins contain similar sequences that could cause cross-reactivity to the immunizations in vivo. A molecular model of the interactions of JAA-F11 to one peptide mimic was constructed, giving insight into how the peptides can structurally act as TF-Ag mimics. Continuing experiments are investigating whether the sera from immunized mice can bind directly to breast tumor cells. Mice that generated antibody reactive to TF-Ag will be challenged with breast tumor cells to determine whether a protective effect was generated in the immunizations.

Overall this research shows that immune responses can be created using peptide mimics of a carbohydrate tumor antigen TF-Ag, which has high, restricted expression on breast tumor cells. These immunizations have implications for clinical use to target TF-Ag positive breast tumors and may be able to block metastasis, the most fatal aspect of this disease. This active immunotherapy approach could decrease the tumor burden in cancer patients by generating a long-lasting response that specifically targets TF-Ag positive cancer cells and blocks metastasis.

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P51-2: A DIRECT SYNERGISTIC EFFECT OF IMMUNOTHERAPY AND CHEMOTHERAPY AS A NEW PARADIGM IN TREATMENT OF BREAST CANCER

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Background: Treatment of patients with advanced stages of breast cancer remains an unresolved clinical problem. New chemotherapeutics resulted in only limited success. Immunotherapy of breast cancer has not yet delivered tangible clinical results. The existing paradigm asserts that combination of immunotherapy and chemotherapy is not an effective approach to cancer treatment due to immunosuppressive effect of the latter. Recently we and others have reported findings from clinical trials, which may challenge that paradigm. Patients with advanced stages of different types of cancer showed high objective clinical response rate to chemotherapy that immediately followed immunotherapy.

Objectives/Hypothesis: These data suggest a new paradigm that vaccination may be most effective in direct combination with chemotherapy. The main objectives of this study are to determine whether immunotherapy sensitizes tumor to chemotherapy and to identify some of the main mechanisms of this effect.

Results: We investigated the possibility of a direct synergy between immunotherapy and chemotherapy in vitro. Tumor-free C57BL/6 mice were immunized with K^b bound

p53-derived peptide. Splenocytes were isolated, re-stimulated with the specific peptide, and then used as effectors in CTL assay against EL-4 target cells loaded with specific (p53) or control peptides. Either splenocytes or EL-4 cells were pretreated overnight with 1.5 mg/mL doxorubicin (DOX) or 12.5 nM paclitaxel (TAX). After overnight incubation, cells were washed and used in CTL assay. Pretreatment of target cells with DOX or TAX significantly increased cytotoxicity. Importantly, that effect was antigen specific since it was observed only in EL-4 cells loaded with specific but not a control peptide. In contrast, pretreatment of splenocytes did not result in enhancement of target cell killing. In parallel experiments we have determined that TAX and DOX both increased the expression of p53 in tumor cells. However, that increase was observed only after 48 hr of treatment and, therefore, could not contribute to observed sensitization of tumor cells to CTLs. To determine the effect of the combined treatment in vivo, mammary carcinoma TUBO was established s.c. in BALB/c mice. This tumor expresses Her2/neu antigen. Five days after tumor injection, mice were split into four groups: (1) control, untreated mice; (2) mice treated with activated dendritic cells (DC) loaded with Her2/neu-derived peptide; (3) mice treated with TAX alone; and (4) mice treated with combination of DC vaccine and TAX. DCs were administered s.c. three times with 7-day interval. TAX was injected i.p. 3 days after the second DC vaccination and again 3 days after the third DC vaccination. DC vaccine alone slowed down tumor growth, which was consistent with previous results obtained by many laboratories. TAX had a similar effect. However, in both cases, tumor growth resumed in about a week after the end of the treatment. In a sharp contrast, tumor size was substantially reduced in mice treated with a combination of DC vaccine and TAX. Most of the mice rejected tumor.

Conclusions: Thus, these data indicate that a direct combination of chemotherapy with cancer vaccine provides substantial antitumor effect via sensitization of tumor cells to CTLs. These experimental models will be used for investigating the mechanisms of this phenomenon.

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P51-3: CARBOHYDRATE MIMETIC PEPTIDES INDUCE TUMOR-ASSOCIATED CARBOHYDRATE-REACTIVE ANTIBODIES IN THE ABSENCE OF PATHOLOGICAL AUTOIMMUNITY

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Purpose: Induction of antibodies against tumor-associated carbohydrate antigens (TACA) raises concerns regarding the balance between antitumor immunity and pathological autoimmunity as TACA can be self-antigens expressed on normal tissue. To demonstrate that autoimmunity is not an inevitable consequence of the generation of carbohydrate reactive antibodies, the immune pathology was analyzed in BALB/c mice immunized with two carbohydrate mimetic peptides that are sufficiently potent to induce antibodies with antitumor reactivity. These two peptides mimic endogenously expressed terminal α -galactose structures on murine tissue. We previously demonstrated that carbohydrate mimetic peptides 106 (GGIYWRDIYWRDIYWRDY) and 107 (GGIYYRYDIYYRYDIYYRYD) mimic complex carbohydrates like LeY and simple mono- and disaccharide, components of a variety of TACA. This multiple antigen mimicry (MAM) provides power to these mimotopes in that they preclude the development of multivalent vaccines encompassing multiple TACA. The mimotopes in one single immunization can induce responses to multiple TACA. Mimotope 107 in particular reacts with the lectin Griffonia simplicifolia lectin I (GS-I) and wheat germ agglutinin (WGA) while mimotope 106 only reacts with WGA. These lectins see terminal monosaccharides.

Experimental Design: Tissues from unimmunized mice were labeled with GS-I and antibody to murine immunoglobulin to compare the expression of terminal galactose residues to the binding pattern of circulating natural antibodies. Antigen specificities of immunized serum antibodies were compared with GS-I lectin by western blots of whole cell lysates from murine mammary 4T1 cells. Tissues from immunized mice were analyzed after 4 immunizations and 1 year later using hematoxylin and eosin stain, TUNEL stain for apoptosis, and Luxol-fast blue staining for myelination. ELISA against ssDNA, dsDNA, and histones was performed on sera from these mice.

Results: Expression of terminal galactose moieties (shown by GS-I lectin binding) is restricted to neurons, endothelial cells, and hematopoietic progenitor cells and closely parallels the immunoglobulin deposition pattern in unimmunized mice. There was no evidence of pathological autoimmunity in any immunized mice. Titers of clinically relevant antinuclear antibodies were not significantly elevated.

Conclusions: These results demonstrate that vaccination with carbohydrate mimetic peptides can enhance antibodies to TACAs without inducing immunopathology.

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P51-4: ANTI-INFLAMMATORY CD4+ REGULATORY CELLS PREVENT MAMMARY CANCER IN MICE

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Background and Objectives: Recent studies suggest that inflammation may be a key contributor to development of breast cancer in women. Scientific and medical data point to immune cells, in particular the balance between pro-inflammatory CD4+ effector (TEFF) cells and anti-inflammatory CD4+ regulatory (TREG) cells, as pivotal mediators in human health and disease. Anti-inflammatory TREG cells inhibit destructive immune responses in both humans and mice; thus, immunotherapy using TREG cells has been proposed to treat inflammatory disorders such as arthritis and inflammatory bowel disease (IBD) in people. TREG cells have been shown to suppress IBD-associated colorectal cancer (CRC) in mice by suppressing tumor necrosis factor- α (TNF- α) and other inflammatory growth factors required to sustain cancer growth. We discovered, during investigations of CRC in ApcMin/+ mice, that adoptive transfer of pro-inflammatory TEFF cells rapidly promotes mammary tumors in the ApcMin/+ mouse model. We hypothesized that the balance between activities of TEFF and TREG cells dictates both autoimmune disease and breast cancer risk in women. Our objective was to use this novel inflammation-driven mouse model to test roles for TREG cells as an innovative and highly effective approach for preventing breast cancer in women.

Methodologies: To assess the potential of TREG cells to prevent or treat inflammation-associated breast cancer, we performed standard adoptive immune cell transfer techniques using female ApcMin/+ mice genetically predisposed to mammary tumorigenesis. For these experiments, 8-week-old female C57BL/6J ApcMin/+ mice received a single dose of 3×10^5 highly purified syngeneic pro-inflammatory CD4+CD45RBhi-CD25- TEFF cells by intraperitoneal (ip) injection, instead of a carcinogen, to induce mammary tumors. This novel model for tumor promotion is attractive because it mimics inflammatory aspects of human disease that are not frequently seen in murine models of breast cancer. Half of the mice (n=10) received a co-transfer of 3×10^5 highly purified syngeneic anti-inflammatory CD4+CD45RBloCD25+ TREG cells ip to assess the ability of TREG cells to suppress inflammatory disease-related tumors. Proof-of-principle was assessed by comparing mammary tumor frequency, tumor multiplicity, and tumor size in TREG cell-treated versus control TEFF recipient mice upon euthanasia at age 12–16 weeks.

Results to Date: We demonstrated a significant increase in mammary tumor frequency after adoptive transfer of TEFF cells in ApcMin/+ mice. Recipients of TEFF cells rapidly developed mammary tumors and thyroiditis but did not develop overt IBD. TEFF cell-triggered development of mammary tumors was dependent upon pro-inflammatory cytokine TNF- α . Co-transfer of TREG cells inhibited TEFF cell-induced mammary tumors in ApcMin/+ mice. Taken together, these data indicate that TREG cells down-modulate destructive inflammatory responses throughout the body and support a unifying hypothesis for immune-mediated disorders in women.

Conclusions: Our data show that anti-inflammatory TREG cells promote systemic immune homeostasis and prevent inflammation-associated cancer. This host protective role for TREG cells in inflammation-associated premalignant events diverges from the paradigm that TREG cells inhibit beneficial host anti-tumor immune responses. Factors that promote favorable activities of TREG cells in suppressing carcinogenic processes may ultimately abolish malignancies of the breast in humans. Targeting deleterious host inflammatory responses may be more effective and less toxic than traditional chemotherapeutic approaches to neoplasia.

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P51-5: IDO INHIBITORS FOR BREAST CANCER: FROM IDEA TO LABORATORY TO MOUSE TO CLINIC

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In early 2002 funding of an IDEA award from the DoD Breast Cancer Program allowed us to initiate preclinical development of a radically new kind of cancer immunotherapy that entered human Phase I clinical trials in fall 2007. This 5-year path from IDEA to clinic started with our findings that drug-like inhibitors of the immune suppressive enzyme IDO could awaken antitumor T cell responses in mice with aggressive HER2 breast cancers, enhancing the efficacy of cytotoxic chemotherapy. Through preclinical evaluation of IDO inhibitors, the study of knockout mice for IDO and Bin1 (an IDO-regulating tumor suppressor), and the discovery of a novel IDO-related enzyme termed IDO2, our findings have seeded the emerging concept that the IDO pathway may be a central driver of immune suppression and malignant progression in cancer (1–6). In 2005, we reported that the Bin1-IDO pathway was critical for tumor growth and that small molecule inhibitors of IDO had potent antitumor properties in the MMTV-neu transgenic mouse model of HER2 breast cancer (1). The development and preclinical evaluation of several classes of IDO inhibitors have since validated the concept that IDO represents an important new therapeutic target (1–4). Mechanistic investigations of a clinical “lead” inhibitor, termed D-1MT, led to discovery of IDO2 may also be involved in therapeutic responses (5,6). Of note, preliminary evidence suggests that inac-

tivating genetic polymorphisms in IDO2 occur commonly in human populations and may be associated with reduced cancer risk, perhaps due to lowered. Genetic studies suggest that IDO contributes to the “smoldering” inflammatory state that facilitates carcinogenesis. IDO dysregulation can drive tumoral immune escape when expressed in tumor cells or in antigen-presenting cells of tumor draining lymph nodes (TDLNs). In breast cancer, studies in transgenic mice and human tumors suggest that the latter mechanism dominates. Significantly, recent work in IDO knockout mice suggest that IDO contributes primarily to invasion and metastasis rather than growth of the primary breast tumor itself, in particular for formation of pulmonary metastases. Thus, IDO inhibitors may be particularly effective against metastatic disease. With Phase I trials of D-1MT initiated in fall 2007, the prospects to take our IDEA from bench to bedside and perform a core test of its utility are now a reality.

1. *Nature Med.* 11, 312-319 (2005).
2. *J. Med. Chem.* 49, 684-692 (2006).
3. *Oncogene*, in press.
4. *J. Med. Chem.*, in press.
5. *Cancer Res.* 67, 792-801 (2007).
6. *Cancer Res.* 67, 7082-7087 (2007).

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P51-6: PASSIVE IMMUNOTHERAPY WITH ANTI-CEACAM6 ANTIBODIES

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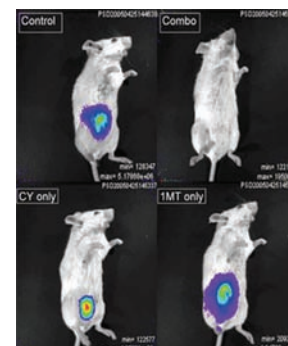
Background and Objectives: Passive immunotherapy using monoclonal antibodies (MAbs) shows promising results for breast tumors. CEACAM6, also called CD66c or NCA-90, is a member of the carcinoembryonic antigen (CEA) family. Expression of CEACAM6 correlates inversely with cellular differentiation and is an independent prognostic indicator of high risk for relapse. Seven out of 10 breast cancer cell lines express significant amounts of CEACAM6. Two anti-CEACAM6 MAbs, MN-3 and MN-15 (Immunomedics Inc., Morris Plains, NJ), which target two different CEACAM6 epitopes, were evaluated for their effects on human breast cancer cells.

Methods: The antiproliferative and chemosensitization effects of the MAbs alone or together with paclitaxel (Taxol®) on breast cell spheroids were evaluated in vitro by spheroid assays using the human breast cancer cell lines MDA-MB-231 (CEACAM6-negative), MDA-MB-468, and MCF-7 (both CEACAM6-positive). The effect of MN-15 on adhesion to extracellular matrix proteins (ECM), adhesion to endothelial cells (MCF-7 only), and invasion through ECM was also determined (MCF-7, MDA-MB-231). MDA-MB-435 (human breast cancer cell line, intermediate expression of CEACAM6) xenograft-bearing mice were treated with MN-15 commencing at the time of implantation of 1×10^7 cells in the mammary fat pad (100 μ g/day times 14 days i.p., then 2 times a week until day 35) or treated with one dose of Taxol (300 μ g, i.p.) alone or combined with MN-15 at day 14.

Results: MN-15 decreased the size (proliferation) and number of breast cancer cell spheroids by 35% and 51%, depending on the cell line. MN-15-treated MCF-7 spheroids were smaller and significantly less numerous than untreated spheroids, and MDA-MB-468 spheroids appeared to be more differentiated. Addition of Taxol (1C50) to antibody treatment did not enhance the effect of the antibody alone. Adhesion to and invasion through ECM was inhibited by MN-15 (MCF-7). MN-15 plus Taxol administered to MDA-MB-435-bearing mice slowed the growth of the primary tumor by 20% ($P < 0.04$ versus Mock-treated controls). However, compared to control antibody (Ag8) + Taxol or Ag8 alone, the results were not significant ($P > 0.05$). Nevertheless, macroscopic pulmonary metastases were observed in the Mock-treated controls, but none were seen in the treatment groups. Cardiac calcification was detected in the combined treatment groups but is nonspecific since it was also observed in the control antibody-treated mice. The reasons for the nonspecific cardiac toxicity need to be investigated.

Conclusion: In vitro, MN-15 reduced the size and number of breast tumor spheroids and inhibited ECM adhesion and invasion. In vivo, MN-15 demonstrated efficacy in reducing the size of orthotopically implanted tumors when combined with Taxol, but more studies are needed to further validate this observation.

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IDO inhibitor 1MT enhances cytotoxic response against 4T1 breast cancer

P51-7: REJECTION OF METASTATIC 4T1 BREAST CANCER BY ATTENUATION OF Treg CELLS IN COMBINATION WITH IMMUNE STIMULATION

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4T1 breast carcinoma is a highly malignant and poorly immunogenic murine tumor model that resembles advanced breast cancer in humans and is refractory to most immune stimulation-based treatments. We hypothesize that the ineffectiveness of immune stimulatory treatment is mediated by the suppressive effects of CD4(+)CD25(+) regulatory T (Treg) cells, which can be attenuated by engaging the glucocorticoid-induced tumor necrosis factor receptor family-related protein with its natural ligand (GITRL); further, combination treatment with existing immune stimulation regimens will augment anti-tumor immunity and eradicate metastatic 4T1 tumors in mice. A soluble homodimeric form of mouse GITRL (mIg-mGITRLs) was molecularly constructed and used to treat orthotopic 4T1 tumors established in immune-competent, syngeneic Balb/c mice. When applied in combination with adenovirus-mediated intratumoral murine granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-12 (IL-12) gene delivery plus systemic 4-1BB activation, mIg-mGITRLs attenuated the immune-suppressive function of splenic Treg cells, which led to elevated interferon-gamma production, tumor-specific cytolytic T-cell activities, tumor rejection and long-term survival in 65% of the animals without apparent toxicities. The results demonstrate that addition of mIg-mGITRLs to an immune-stimulatory treatment regimen significantly improved long-term survival without apparent toxicity and could potentially be clinically translated into an effective and safe treatment modality for metastatic breast cancer in patients.

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P51-8: GENETICALLY ENGINEERED T-CELLS FOR ADOPTIVE IMMUNOTHERAPY OF BREAST CANCER

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We have pioneered and developed the "T-body" approach in which the patient's own T and natural killer (NK) cells are redirected with antibody specificity to fight his/her cancer. T-bodies are effector lymphocytes genetically modified to express chimeric receptors composed of antibody recognition unit in the form of antibody-derived single chain variable fragment (scFv) linked to intracellular co-stimulatory and stimulatory domains of T-cells' receptor (CR) chains. Following to the expression of such cancer-specific tripartite CR, T-bodies are geared to undergo full activation in non-MHC dependent or restricted manner and eliminate the cancer target. Under the BCRP support, we have focused on the generation of CR specific to the otherwise incurable advanced stage of cancer-bone metastases. We have chosen to redirect the T-bodies to HER2/Neu that is overexpressed in a large fraction of breast adenocarcinoma patients and on vast majority of metastatic cells of this cancer. First we have demonstrated the ability of human peripheral blood lymphocytes endowed with HER2/Neu specificity to recognize and eliminate established human breast cancer xenografts growing subcutaneously or orthotopically in the murine mammary gland. Intratumoral administration of the T bodies were effective in the rejection of relatively large volume of tumors. To obtain a therapeutic effect of primary as well-disseminated tumors following to a systemic administration of T-bodies, the mouse "patients" have to be pretreated by mild lymphoablative regimens of irradiation and/or cyclophosphamide. IL-2, as well as other homeostatic interleukines (e.g., IL-7 and IL-15) help to improve the persistence and anti-tumor effect of the T-bodies in vivo. Later on we have studied and demonstrated the ability of human and murine T-bodies to prevent the development and eliminate spontaneous mammary tumors expressing HER2/Neu in transgenic mice. Transgenic mice, expressing HER2/Neu-specific chimeric receptors that we have generated serve as a source for naïve effector cells expressing cancer-specific CR. Their comparison to ex vivo genetically engineered T-bodies provided us with valuable information on how to optimize the redirected for better therapy in patients. Taken together, our studies have provided a proof of concept in animal models that is followed up for the preparations of improved protocols for clinical trials that take place now in several centers around the world.

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P51-9: RETINOIC ACID AND ALPHA-GALACTOSYLCERAMIDE, A LIGAND FOR CD1d ON ANTIGEN-PRESENTING CELLS, DIFFERENTIALLY REGULATE THE PRODUCTION OF IMMUNOREGULATORY CYTOKINES BY CULTURED DENDRITIC CELLS AND SPLENOCYTES

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Background and Objectives: Activation of the natural immune system is promising as a way to inhibit tumor growth. Dendritic cells (DC), natural killer (NK) cells, and a specialized subset of T cells known as iNKT cells are among the cell types likely to inhibit tumor growth in vivo. Based on previous results, we postulated that a combination of retinoic acid (RA), an agent that often inhibits cell growth and induces cell differentiation and which can induce the expression of CD1d by antigen-presenting cells, may, in the presence of alpha-galactosylceramide (GalCer) which binds to CD1d, augment the activation of iNKT cells. In animals, GalCer has shown encouraging results against several types of cancer, but to our knowledge, it has not been tested in combination with RA or agents such as poly-I:C, a strong inducer of interferons (IFN). We thus have proposed that a triple nutritional-immunological combination of RA, GalCer, and poly-I:C could be effective for breast cancer immunotherapy based on the ability of: (1) RA to induce CD1d, (2) GalCer to bind to CD1d on antigen-presenting cells (dendritic cells, DC, or macrophages) and activate iNKT cells, and (3) poly-I:C to stimulate the production of cytokines that activate NK cells. Our research goal is to test whether these agents may synergistically inhibit breast tumor growth in mice. In the first 6 months of our award, we have examined whether RA together with GalCer regulates the proliferation of DC and splenocytes in culture and their ability to produce immunoregulatory cytokines.

Methods: DC were prepared from bone marrow of the femur and tibia of adult (>8 wk) female Balb/c or C57BL/6 mice. GM-CSF was added to the cultures in complete media every 3 days for a total of 9 days to induce DC growth. On day 9, TNF (5 ng/mL) was added to induce DC differentiation. Splenic mononuclear cells were treated with RA and/or GalCer for 24 h. To monitor NKT cell proliferation, differentiated DC or isolated splenocytes in 96-well plates were treated with RA (20 nM) or GalCer (100 nM) for 24 h. The inactive anomer, beta-GalCer (100 nM), was used as control. Two NKT tumor cell lines were then added to the DCs or splenocytes for 48 h as potential responders to the presentation of GalCer bound to CD1d. For the last 4 h, 3H-thymidine was added to monitor cell proliferation.

Results to Date: (1) RA significantly reduced the proliferation of the two NKT tumor cell lines, DN32.2 and TCB11. Proliferation was also decreased in co-cultures of NKT cells with DC but not with spleen cells. (2) Spleen cells secreted IL-4 and IFN-γ only when cultured with GalCer. The presence of the NKT tumor cells further increased spleen cell cytokine production although the NKT cells alone produced neither cytokine. (3) The production of IL-4 and IFN-γ by spleen cells was differentially regulated by RA as RA increased the output of IL-4 when GalCer-activated spleen cells were co-cultured with NKT cells, but at the same time RA reduced the output of IFN.

Conclusions: The growth-inhibitory effects of RA on the NKT tumor cells is encouraging, but further in vivo studies are needed. GalCer markedly induced IFN production by splenocytes. The attenuation by RA needs to be further evaluated and poly-I:C added in the triple stimulation model.

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P51-10: ANTITUMOR ACTIVITY OF γδ T CELLS EXPANDED FROM PERIPHERAL BLOOD OBTAINED FROM PATIENTS WITH METASTATIC BREAST CANCER WHO ARE ACTIVELY UNDERGOING THERAPY

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Background and Rationale: Cancer cells can express stress-induced self antigens that while neither tumor-specific nor tumor-derived can nonetheless serve as recognition determinants for γδ-T cells. Such observations have contributed to the emerging view that γδ-T cells might provide protective immunosurveillance against various malignancies. We have previously identified a physiological signaling pathway that inhibits apoptosis in mitogen-stimulated human γδ-T cells. This now permits us to expand large numbers of human peripheral blood-derived γδ-T cells that retain innate antitumor activity against a wide variety of human tumor cell lines, including breast cancer cells. Importantly, this serves as the rationale for designing clinical strategies to expand then reinfuse patient-derived γδ-T cells as a new form of cellular immunotherapy for breast

cancer. It has been recently reported that patients with a variety of cancers may have reduced numbers of circulating $\gamma\delta$ -T cells and that these cells may also display diminished function. Furthermore, it is not known to what extent tumor-reactive $\gamma\delta$ -T cells can be expanded and isolated from peripheral blood obtained from patients who are actively undergoing treatment, such as chemotherapy.

Findings: As in the case of healthy donors that we have previously published, here we show that apoptosis-resistant $\gamma\delta$ -T cells can likewise be expanded ex vivo from peripheral blood obtained from patients with metastatic breast cancer who are actively undergoing therapy. In short-term cultures, expansion of apoptosis-resistant $\gamma\delta$ -T cells derived from patients with breast cancer is less robust (73-fold mean expansion) compared to expansion of apoptosis-resistant $\gamma\delta$ -T cells derived from healthy donors (over 400-fold mean expansion). Nevertheless, despite the lower expansion efficiency of apoptosis-resistant $\gamma\delta$ -T cells obtained from patients with breast cancer who are actively undergoing therapy, these $\gamma\delta$ -T cells retain innate cytolytic activity against a number of human breast cancer cell lines, but fail to lyse nonmalignant normal human fibroblast control cells.

Significance and Conclusions: These studies demonstrate the feasibility of expanding $\gamma\delta$ -T cells from patients with metastatic breast cancer (who are actively undergoing therapy) and serve to emphasize the importance of optimizing the methods for the ex vivo expansion of human $\gamma\delta$ -T cells. Importantly, in these studies, $\gamma\delta$ -T cells were expanded in cultures using the clinical-grade reagents that are to be used in generating $\gamma\delta$ -T cells for use in the filing of an Investigational New Drug (IND) application with the U.S. Food and Drug Administration. Thus, our demonstration that it is indeed possible to expand breast cancer-reactive $\gamma\delta$ -T cells from patients with metastatic breast cancer who are actively undergoing therapy suggests that Phase I clinical trials can be designed to include such patients with advanced disease.

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P51-11: IMMUNOTHERAPY OF SPONTANEOUS MAMMARY CARCINOMAS IN A MURINE MODEL DEFICIENT FOR TELOMERASE ACTIVITY

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Telomere maintenance and telomerase are implicated in the development, progression, and metastasis of malignant tumors including breast cancer. To determine the role of telomerase in the development of mammary carcinomas, we generated telomerase knockout mice (mTERC^{-/-}) mice that express polyomavirus middle T antigen (PyMT) and MUC1 tumor-associated antigen for consecutive generations. The telomere lengths are progressively shortened in each successive generation of mTERC^{-/-}MMT mice. More important, tumor development and tumor volume are inversely correlated with the generation of mTERC^{-/-}MMT mice. In the present study, we aim to assess the effect of telomerase inactivity on antitumor immunity induced by the fusions of dendritic cells and MUC1-positive tumor cells (FC/MUC1). MMT, heterozygotes (mTERC^{+/-}) MMT mice (G0) and first (G1) to third (G3) generations of mTERC^{-/-}MMT mice were immunized with FC/MUC1 when the mice were 3–4 weeks of age. In naïve mice, there is minimal CTL activity against MUC1-positive tumor cells. In contrast, vaccination of MMT and G0 to G3 mTERC^{-/-}MMT mice induced MUC1-specific CTL. The CTL activity in MMT and G0 to G3 mTERC^{-/-}MMT mice against MUC1-positive tumor cells is 21%, 22%, 23%, 19%, and 14%, respectively, suggesting that the cellular immunity is not affected by telomerase inactivity, at least in the G1 and G2 mTERC^{-/-}MMT mice. The induction of CTL in these mice translated into delayed appearance of mammary carcinomas. The latent time for the mammary tumors in MMT, G0 to G3 mTERC^{-/-}MMT mice immunized with FC/MUC1 were 113.7 ± 9.3, 119 ± 9.94, 126.4 ± 15.8, 136.5 ± 15.8, and 152 ± 20.4 days, respectively. To determine whether telomerase inactivity has a detrimental effect on T cell proliferation or cell division, we first measured the T cell proliferation in mice with or without FC/MUC1 immunization using standard isotope incorporation. Minimal T cell proliferation was observed in lymph node cells (LNC) or splenocytes from non-immunized mice. In contrast, LNC and splenocytes from MMT, G0 and G1 mTERC^{-/-}MMT mice immunized with FC/MUC1 proliferated vigorously. We next labeled T cells with the fluorescent dye carboxyfluorescein diacetate succinimidyl diester (CFSE) and measured T cell division at multiple time points. Both CD4 and CD8 T cells from immunized MMT mice divided vigorously. Similar results were obtained in CD4 and CD8 T cells from immunized G0 to G2 mTERC^{-/-}MMT mice. Multiple rounds of cell division were observed in both CD4 and CD8 T cells from immunized G0 to G2 mTERC^{-/-}MMT mice. In contrast, fewer rounds of T cell divisions were observed from G3 mTERC^{-/-}MMT mice compared with those from G1 and G2 mTERC^{-/-}MMT mice. Taken together, these results indicate that the ability of T cell proliferation or division is not affected in the early generations of mTERC^{-/-}MMT mice. However, decline of T cell division is observed in G3 mTERC^{-/-}MMT. Based on these preliminary results, we conclude that telomerase inactivity enhances the antitumor immunity, at least in the first and second generations of mTERC^{-/-}MMT mice. These results may lead to a novel approach of management of breast cancer based on combined anti-telomerase and other therapies.

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P51-12: LYMPHOTROPIC CYTOKINE IMMUNE COMPLEXES PROMOTE EFFECTIVE ANTI-TUMOR AUTOIMMUNITY IN A MODEL OF METASTATIC BREAST CARCINOMA

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We have previously shown that lymphopenia-induced homeostatic T cell proliferation promotes anti-tumor autoimmunity. However, our results indicated that lymphopenia may also facilitate metastasis dissemination. These findings prompted us to explore alternative means to induce T cell activation and their applicability in cancer immunotherapy. Based on the observation that the in vivo effect of IL-2 is dramatically enhanced if this cytokine is administered as a complex with specific antibodies (Ab), we examined whether the same was applicable to IL-7, thought to be a main factor in driving homeostatic proliferation. Whereas IL-7 or IL-2 injected alone had no effects, immune complexes of these lymphotropic cytokines significantly enhanced proliferation and accumulation of CD4 and CD8 T cells in non-lymphopenic mice. Importantly, in mice challenged with highly metastatic 4T1 breast carcinoma cells, IL-7/Ab and in particular IL-2/Ab complexes significantly reduced lung metastasis and extended survival. These immune complexes were effective therapeutically against established tumors and were dependent on CD8 T cell activation. Additional studies with T cell receptor-transgenic cells showed that IL-2/Ab complexes induce proliferation, promote activation of cytolytic effector functions, and reverses anergy in antigen-specific CD8 T cells. Thus, immune complexes of lymphotropic cytokines are more effective than homeostatic proliferation in inducing T cell expansion, activation, break of tolerance, and therapeutic anti-tumor responses in a model of aggressively metastatic breast carcinoma.

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P51-13: TOLERANCE SUPPRESSION THERAPY AS A MEANS OF ENHANCING TUMOR VACCINES

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The ability of the immune system to specifically recognize antigens makes it a potentially powerful tool for developing modalities to treat cancer. In spite of the identification of numerous tumor-specific antigens and the development of potent tumor vaccines, immunotherapy has yet to live up to its full potential. In part this is due to the ability of tumors to evade the immune response by inducing tumor-antigen-specific tolerance.

Our laboratory is interested in determining the pathways that promote full T cell activation and T cell tolerance. Through high dimensional transcriptional analysis, we have identified several TCR-induced pathways that lead to the induction of T cell tolerance. We reasoned that pharmacologic inhibition of these pathways in combination with tumor vaccines might enhance the efficacy of anti-tumor immunotherapy—*Tolerance Suppression Therapy*. Among the pathways identified was TCR-induced PKC activation. Indeed, our lab shows that a PKC inhibitor, Gö6976, can block tolerance induction, but not T cell activation, in vitro. Based on these observations, we hypothesized that adding Gö6976 to existing murine tumor vaccine protocols could enhance the protective effect of the vaccines. Our studies demonstrate that in the Her-2/neu model of spontaneous breast cancer the addition of Gö6976 to a vaccine could enhance the anti-tumor immune response and modestly improve survival. A second pathway identified was the ability of adenosine to promote tolerance by stimulating the A2aR on activated T cells. We have been able to show that A2aR stimulation promotes T cell anergy and the induction of regulatory T cells. In as much as the concentration of adenosine in the tumor micro environment is elevated, we hypothesized that tumor-derived adenosine acting via the A2aR on T cells could promote tumor-induced tolerance. In support of this hypothesis and consistent with data from the Sitkovsky group (1), we are able to demonstrate that A2aR null mice are more resistant to tumor challenge in multiple tumor models. Furthermore, T cells from A2aR null mice generate increased memory responses to vaccines and are more effective in protecting mice against tumor challenge. These findings suggest that A2aR antagonists might prove to be potent adjuvants to tumor vaccine therapy.

Overall, our data suggest that pharmacologic suppression of tolerance during immunotherapy is a potentially potent mechanism to enhance the ability of tumor vaccines to eradicate tumors.

1. *Proc Natl Acad Sci USA*. 2006 Aug 29;103(35):13132-7

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P51-14: DEVELOPMENT OF T CELL-BASED IMMUNOTHERAPIES AGAINST BREAST CANCER

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The aim of our studies was to develop novel T cell immunotherapies against breast cancer. The studies were based on reports demonstrating a positive correlation between T lymphocytic infiltration of these tumors and favorable clinical outcome. The major goal of the proposed studies was to isolate and characterize cytolytic T lymphocytes (CTL) with in vivo like T cell receptors. The CTL provide the basis for adoptive CTL immunotherapy and active immunotherapy with CTL-derived peptides/antigens. Thirty breast carcinoma tissues were cultured in organotypic cultures (reconstructs) and/or mixed lymphocyte/tumor cultures. T cell lines were established from 25 breast cancer specimens. Four breast cancer cell lines were also established. Cytolytic activity was demonstrated in 21 of 25 T cell lines tested. Two of 9 tested T cell lines were shown to have helper function. The T cell lines may be used for adoptive immunotherapy and antigens recognized by the T cells for active immunotherapy of breast cancer patients.

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P51-15: USE OF PHYTOCOMPOUNDS AND GENE-BASED VACCINE APPROACHES AGAINST TUMOR MALIGNANCY

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Background: This study was pursued as a continuation of the original initiative of the DAMD 17-96-2-6017 breast cancer research grant project (Awardee: Ning-Sun Yang, 1998–2002). Two approaches were taken for our studies; the first was on evaluating the effect of specific phytochemicals on anti-breast cancer cell activities and the second one is a study for candidate breast tumor vaccines. For chemo-preventive phytochemical study, we evaluated the possible mechanism(s) with which taiwanin A, a major ligand isolated from *Taiwania cryptomerioides*, inhibits the growth of human MCF-7 mammary carcinoma cells. To enhance anti-tumor effect with the designed gene-based cancer vaccines, we investigated the role and possible use of a chemokine transgene (mRANTES) co-delivered with a tumor-associated antigen (TAA) gene (hgp100 cDNA), followed by boosting with a modified vaccinia virus ankara (MVA) vector expressing the same transgenes.

Methods: A number of assay systems including TUNEL assay, western blotting, RNAi approach, and reporter gene assay were employed to characterize the anti-MCF-7 cells activity of taiwanin A and their molecular mechanisms against MCF-7 cells. Mouse skin tissues were transfected with mRANTES cDNA at different times; hgp100 was gene gun-delivered before or/and after mRANTES application and boosted with an MVA vector expressing both genes.

Results: Taiwanin A was found to time dependently induce ROS activity, which may activate ATM and Chk activities. Taiwanin A could also up-regulate the expression of p53, p-p53, p21(Cip1), and p27(Kip1), and down-regulate the levels of G₂/M checkpoint Cdk1-cyclin A/B, leading to potentiation of G₂/M cell-cycle arrest in MCF-7 cells. siRNA approach demonstrated that the cell-cycle arrest induced by taiwanin A was p53-dependent and reversible. Furthermore, the FasL/Fas mediated apoptotic signaling cascade, not the mitochondria-initiated pathway, was found to involve in taiwanin A-induced apoptosis via the activation of caspases-10 and -7 activities, and the proteolytic cleavage of PARP. With the second approach, we show that the above "heterologous prime-boost" vaccines strongly suppressed the subcutaneous growth of B16/hgp100 tumors and their metastasis to lungs. With the vaccination regimen mRANTES(Oh)+hgp100(24h), tumor growth was significantly lower than with hgp100 alone and enhanced mouse survival rate. It also increased the splenocytes killing activity of B16/hgp100 cells. B16/hgp100 melanoma cells were found as resistant to TRAIL and FasL in vitro but were sensitized to the ligands in vivo owing to the priming effect of cytokines in response to vaccination.

Conclusions: These results demonstrate that taiwanin A can modulate highly specific molecular/cellular signaling mechanisms associated with anti-breast cancer activities, suggesting that taiwanin A may be a good candidate for future development as anti-breast cancer agent. Co-vaccination of mRANTES with TAA gene(s) at a specific time effectively suppressed tumor growth and lung metastasis in test mouse model. Our findings suggest that specific phytochemicals and chemokine studied in this project may warrant future investigations to combat the breast tumor growth and metastasis.

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P51-16: CD4+ Th1 HER2-SPECIFIC T CELLS AS A NOVEL TREATMENT FOR HER2-OVEREXPRESSING BREAST CANCER

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Adoptive T cell therapy for solid cancers has shown promise in preclinical models but limited clinical responses in patients. To date, many strategies have focused on the use of CD8+ cytotoxic T cells to mediate tumor regression. We hypothesized that antigen-specific CD4+ T cells may be therapeutically effective because: (1) their secretion of proinflammatory cytokines can modulate the tumor microenvironment and enhance endogenous antigen presentation by antigen-presenting cells (APC), and (2) provision of a robust CD4+ Th1 (type helper 1) cell response should activate tumor-specific CD8+ T cells endogenously. We have previously reported that peptide vaccination increases the frequency of peptide-specific T cells, which can then be harvested for ex vivo T cell expansion. Using a murine model of HER2-mediated breast cancer, the neu-transgenic mice [FVB/N-TgN(MMTV^{neu})-202Mul], we identified several immunodominant CD4+ T helper epitopes of HER2/neu: p101 (RLRIVRGTLQFEDKYAL), p373 (KIFGSLAFLPES FDGDPS), p45 (HLDMLRHLYQGCQVV), and p780 (GVGSPYVSRLLGICL). Each peptide-reactive T cell line (derived from spleens of immunized mice) demonstrated peptide specificity in IFN- γ enzyme-linked immunospot (ELISPOT) assays. Furthermore, the T cell lines inhibited tumor growth in mice bearing neu+ tumors; at day 21 of the study (i.e., 11 days after the first of 3 T cell infusions), mice that received peptide-specific T cells showed an approximate 60% reduction in tumor size compared to mice treated with control (naïve) T cells. Infusion of T cells specific for the 3 most immunogenic peptides (p101, p373, and p45) led to an even greater inhibition, reducing tumor size by 81% (p=0.0423). Furthermore, survival of treated mice was significantly enhanced compared to control mice (100% versus 0%, p=0.0208). Tumor rejection involved endogenous CD8+ T cells since depletion of these cells in vivo before or during T cell therapy abrogated tumor rejection. Depletion of endogenous CD4+ T cells and B cells in vivo had no effect on tumor rejection. Multiplex cytokine assay revealed that during ex vivo culture, when stimulated with antigen, the HER2-specific CD4+ T cells (compared to unstimulated or nonspecific T cells) secreted high levels of Th1 cytokines (e.g., IFN- γ , GM-CSF) as well as IL-17. In conclusion, we have identified native MHC class II epitopes of HER2/neu in the neu-transgenic mice and demonstrated the unique use of CD4+ Th1 HER2/neu-specific cells for the treatment of neu-expressing tumors, predominantly through the potent activation of CD8+ T cell-mediated immunity.

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P51-17: TGF β BLOCKING AGENTS FOR THE IMMUNOTHERAPY OF BREAST CANCER

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At late stages of cancer progression, breast cancer cells secrete high levels of transforming growth factor beta protein (TGF β) that acts as a potent promoter of tumor progression and metastasis. Previous studies have shown that TGF β receptor II (T β RII) inactivation reduces the metastasis of high-grade tumors. Similarly, treatment of tumor-bearing mice with a soluble form of T β RII protein was able to block tumor metastasis in mouse models of breast cancer. However, tumor cell proliferation and neoangiogenesis associated with the tumor were not affected by soluble T β RII treatment. In the present study, we aim to combine the antimetastatic effect of soluble T β RII protein with the induction of a potent antitumor response mediated by proinflammatory cytokines such as IL-2 and GM-CSF. We have generated two chimeric proteins composed of IL-2 or GM-CSF and soluble T β RII. Such chimeric proteins are endowed with the ability to block TGF β -dependent effects and induce an immune activation against breast cancer cells, which may result in regression and clearance of a vastly bulkier pre-established native cancer.

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P51-18: DISRUPTION OF TGF- β SIGNALING USING A SMALL MOLECULE TGF- β RI ANTAGONIST IMPROVES THE EFFICACY OF DENDRITIC CELL VACCINES FOR BREAST CANCER**Matthew Peter Rausch**

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Despite advances in cancer therapy, breast cancer remains the second leading cause of cancer-related death among American women. Existing therapies, such as surgery, radiation, and chemotherapy, are limited in their ability to control cancer spread and recurrence and are associated with severe toxicity. Given these limitations, immunotherapy has been proposed as an alternative to existing cancer therapeutics. Immunotherapy stimulates the body's immune system to fight cancer in the same way that it fights the common cold and offers several advantages over conventional therapeutic approaches. One form of immunotherapy involves the use of dendritic cells (DCs). DCs are immune cells that patrol the body searching for signs of infection or cancer. When infections or tumors are detected, DCs take up pieces of infected or abnormal cells called antigens, present them to T cells, and stimulate T cell responses. T cells activated by DCs go on to kill the infected or abnormal cells. DC vaccination has shown promise in animal studies but has demonstrated limited clinical success in

humans. The failure of DC vaccines in humans has been attributed in part to suppressive factors produced by the tumor that inhibit the ability of the immune system to mount effective immune responses. One such factor is transforming growth factor beta (TGF- β). TGF- β impairs the ability of DCs to take up antigen and stimulate T cells. SM16 is a novel drug that blocks the effects of TGF- β and therefore represents a promising strategy to prevent TGF- β -mediated immunosuppression in the context of DC vaccination for cancer. In this study, we evaluated the ability of SM16 to improve the efficacy of DC vaccines to treat primary and metastatic breast tumors in mice. Our results show that SM16 prevents Smad2 phosphorylation, an early step in the TGF- β signaling cascade in DCs and cultured 4T1 tumor cells in vitro and primary and metastatic 4T1 tumor tissue in vivo. In addition, i.p. administration of SM16 or SM16 in combination with DCs moderately inhibited the growth of primary and metastatic 4T1 tumors in vivo. SM16 was well tolerated and mice receiving the drug did not demonstrate any overt signs of toxicity. Furthermore, oral administration of SM16 alone or in combination with DCs also inhibited the growth of primary and metastatic 4T1 tumors. Taken together these data show that SM16 is an effective treatment for primary and metastatic 4T1 breast tumors and that this drug may be a viable strategy to overcome TGF- β -mediated immunosuppression in the context of DC vaccination.

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TUMOR IMMUNOLOGY

Poster Session P52

P52-1: REGULATION OF THE MIGRATION OF FoxP3+ REGULATORY T CELLS INTO LYMPHOID TISSUES AND BREAST TUMORS

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Background and Objectives: Forkhead box P3 (FoxP3)-positive T cells are a specialized T cell subset for immune regulation and tolerance (Kim CH. *Mini Rev. Med. Chem.* 2007, 11:1136-43). FoxP3⁺ T cells are greatly enriched in breast tumors (Bates et al. *J. Clin. Oncol.* 2006 24:5373-80), and it is thought that tumors harbor FoxP3⁺ T cells to evade host immune responses. In contrast to this view, it has been reported also that FoxP3 expressed in breast tumor cells is an X-linked breast cancer suppressor gene and an important regulator of the HER-2/ErbB2 oncogene (Zuo et al. *Cell.* 2007, 129:1275-86). Whichever the case, expression of FoxP3 and cells that express this transcription factor are important for the pathogenesis of breast tumor cells. It is poorly understood how FoxP3⁺ T cells migrate into tumors. Understanding this process would provide a useful therapeutic target to promote host immune responses to the tumors.

Hypothesis and Methodologies: It has been established that T cells including FoxP3⁺ T cells undergo trafficking receptor switches to migrate from organ to organ (Kim CH. *Curr. Opin. Hematol.* 2005, 12:298-304; Kim CH. *Exp. Hematol.* 2006 34:1033-40). We hypothesize that a similar mechanism of trafficking receptor switch would be involved for generation of FoxP3⁺ T cells that effectively migrate to tumors. We performed trafficking receptor analysis and in vitro and in vivo homing studies in mouse breast tumor models utilizing wild-type and trafficking receptor-deficient FoxP3⁺ T cells.

Results to Date: As the first step, we investigated the trafficking receptor switches of FoxP3⁺ T cells in thymus and secondary lymphoid tissues and the functional consequences of these switches in migration. We found that FoxP3⁺ T cells undergo two discrete developmental switches in trafficking receptors to migrate from primary to secondary and then to nonlymphoid tissues in a manner similar to conventional CD4⁺ T cells as well as unique to the FoxP3⁺ cell lineage. In the thymus, precursors of FoxP3⁺ cells undergo the first trafficking receptor switch (CCR8/CCR9-->CXCR4-->CCR7), generating mostly homogeneous CD62L⁺CCR7⁺ FoxP3⁺ T cells. Consistent with this switch, recent FoxP3⁺ thymic emigrants migrate exclusively to secondary lymphoid tissues but poorly to nonlymphoid tissues or tumors (Lee JH et al. *J. Immunol.* 2007;178:301-11). Next, we investigated migration of FoxP3⁺ T cells into nonlymphoid tissues versus breast tumors (4T1 tumors established in BALB/c mice). The FoxP3⁺ thymic emigrants undergo the second switch in trafficking receptors for migration to nonlymphoid tissues and breast tumors upon Ag priming. This second switch involves downregulation of CCR7 and CXCR4 but upregulation of a number of memory/effector-type homing receptors, resulting in generation of heterogeneous FoxP3⁺ T cell subsets expressing various combinations of trafficking receptors including CCR2, CCR4, CCR6, CCR8, and CCR9 (Lim et al. *J. Immunol.* 2006, 177:840-51). A notable difference between the FoxP3⁺ and FoxP3⁻ T cell populations is that FoxP3⁺ T cells undergo the second homing receptor switch at a highly accelerated rate compared with FoxP3⁻ T cells, generating FoxP3⁺ T cells with unconventionally efficient migratory capacity to major nonlymphoid tissues and breast tumors.

Conclusions: Our results revealed the trafficking receptor requirement for migration of FoxP3⁺ T cells into normal organs and breast tumors.

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P52-2: INFLAMMATION INDUCES TUMOR GROWTH BY NUCLEAR-FACTOR- κ B SIGNALING IN MYELOID-DERIVED SUPPRESSOR CELLS

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Myeloid-derived suppressor cells (MDSC) are potent inhibitors of anti-tumor immunity that facilitate tumor progression by blocking the activation of CD4⁺ and CD8⁺ T cells and by promoting a type 2 immune response through their production of IL-10 and down-regulation of macrophage production of IL-12. Because they accumulate in many cancer patients, MDSC are a significant impediment to active cancer immunotherapies. Chronic inflammation has recently been shown to enhance the accumulation of MDSC and to increase their suppression of T cells. These findings led us to hypothesize that inflammation contributes to tumor progression through the induction of MDSC that create a favorable environment for tumor growth. Since chronic inflammation also drives type 2 immune responses that favor tumor growth, we have now asked if inflammation mediates this latter effect through MDSC. We find that inflammation increased IL-10 production by MDSC and induces MDSC that are more effective at down-regulating macrophage production of IL-12 as compared to MDSC isolated from less inflammatory tumor micro-environments, thereby skewing tumor immunity toward a type 2 response. In vitro experiments using bacterial lipopolysaccharide demonstrated that MDSC phenotype is induced by signaling through the TLR4-

NF κ B pathway and involves up-regulation of CD14. Although NF κ B is well-recognized as a key regulatory protein in other myeloid cells, it has not previously been implicated in MDSC function. These studies demonstrate that MDSC are an intermediary through which inflammation promotes type 2 immune responses, and they identify a novel mechanism by which activation of NF κ B downregulates tumor immunity and enhances tumor growth.

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P52-3: ENHANCEMENT OF THE IMMUNE RESPONSE FOLLOWING IMMUNIZATION WITH A MUC1-C3d FUSION PROTEIN

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MUC1 is a transmembrane glycoprotein normally expressed on the apical surface of epithelial cells. However, on many types of cancer cells MUC1 is aberrantly glycosylated and overexpressed in a depolarized fashion, making MUC1a potential target for immunotherapy. Patients with MUC1 positive cancers often have a low titer IgM and a low frequency CTL response against MUC1. We used MUC1 transgenic mice (MUC1tg), which display a similar response to MUC1 as seen in human, to investigate a vaccination strategy to enhance MUC1 immunogenicity. Complement is known to play an important role in modulating humoral immunity, and the binding of the complement activation product C3d to CR2 on B cells provides a costimulatory signal when C3d is linked to antigen. More recent evidence shows that complement can also modulate T cell immunity. Fusion proteins consisting of antigen linked to repeating units of C3d have been shown to be effective molecular adjuvants that can significantly enhance an antibody response. We investigated whether a MUC1-C3d fusion protein could enhance MUC1 immunogenicity and break tolerance to MUC1 in MUC1tg.

Protein constructs containing either 5 tandem repeats of MUC1 linked to 3 repeating units of C3d (MUC1/TR5-C3d) or 5 tandem repeats of MUC1 only (MUC1/TR5) were prepared. MUC1tg (C57BL/6) were immunized intramuscularly with either construct and boosted on days 28 and 69. Antibody response was analyzed by ELISA and T cell response analyzed by interferon gamma ELISPOT using MUC1 transfected EO771 cells as targets (EO771 cell line was derived from a spontaneously arising breast tumor in C57BL/6 mice). MUC1 transgenic mice treated with either MUC1/TR5 or MUC1/TR5-C3d had both IgG and IgM anti-MUC1 present in their sera at day 28. However, while both groups had similar IgM responses, mice vaccinated with MUC1/TR5-C3d had significantly higher IgG titers at days 28, 35, and 78. More unexpectedly, MUC1/TR5-C3d elicited a strong T cell response to EO771/MUC1+ cells compared to only a very weak T cell response in mice immunized with MUC1/TR5. A DNA vaccination approach failed to elicit an immune response.

Thus, C3d functions as a molecular adjuvant when linked to a cancer-associated self-antigen and may help break tolerance in cancer patients. Studies are under way to determine the specificity of the C3d-enhanced immune response and whether it is protective in a mouse model of cancer.

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P52-4: PROGRAMMED CELL DEATH INDUCED BY c-FLIP-DRIVEN PEPTIDE: A NOVEL CANCER THERAPEUTIC STRATEGY

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The cellular FLICE inhibitory protein (c-FLIP) is one of the major regulators of death ligand-induced apoptosis. Both of the splicing isoforms, c-FLIPL and c-FLIPS, have been reported to inhibit tumor necrosis factor (TNF) family cytokine-mediated apoptosis in certain cancer cells. Previously, we reported that c-FLIPL interacts with death receptor 5 (DR5) in a FADD-independent manner, resulting in an inhibition of spontaneous apoptosis by TNF-related apoptosis-inducing ligand (TRAIL). Herein, we show that the c-FLIPL-driven cellular permeable TAT-FLIP peptide triggers apoptosis and inhibits tumor growth in vivo. The binding of the TAT-FLIP peptide to DR5 initiates c-FLIPL release and processing from DR5 and the recruitment of FADD to DR5 simultaneously. The release of c-FLIPL results in a reduction of the interaction between c-FLIPL and p85, which leads to the inhibition of c-FLIPL-dependent Akt phosphorylation. Moreover, pretreatment with the TAT-FLIP peptide sensitizes tumor cells to TRAIL-induced apoptosis. These results provide new insights into understanding the role of c-FLIP variants in death receptor-induced death signaling and their potential as targets for cancer therapy.

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P52-5: β 4-INTEGRIN AS A MOLECULAR MARKER AND A RESPONSE PREDICTOR IN CANCER THERAPY

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TNF-Related Apoptosis-Inducing Ligand (TRAIL) belongs to the TNF family of cytokines. TRAIL selectively induces cell death in tumor/transformed cells but not in normal cells. β 4-integrin is a transmembrane glycoprotein that mediates interaction between the extracellular matrix (ECM) and the cells and is mainly involved in cell proliferation, adhesion, migration, and survival. Here we show that the expression of β 4-integrin is significantly reduced in TRAIL-resistant MDA-MB-231/TR and SUM159/TR cells at both mRNA and protein levels. Knock-down of β 4-integrin by RNA interference significantly reduces TRAIL sensitivity in MDA-MB-231 cells. Indeed, the introduction of β 4-integrin to β 4-integrin-negative cells sensitizes both carcinoma cells to TRAIL-induced apoptosis through interaction with death receptors 4 and 5. Moreover, the disruption of interaction between β 4-integrin and death receptor by point mutation at the 659 amino acid site in β 4-integrin attenuates β 4-integrin-dependent TRAIL sensitization. Furthermore, the expression of β 4-integrin is well correlated with TRAIL sensitivity in breast and prostate cancer cells. Our results indicate that β 4-integrin can be used as a predictor and an "amplifier" of TRAIL chemotherapy.

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P52-6: IMMUNOTHERAPY AGAINST METASTATIC BREAST CANCER WITH A TWIST

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Background: Effective loco-regional treatments are available for breast cancer, but metastatic disease remains a therapeutic challenge. The effectiveness of immunotherapy (IT) will depend on the ability to target antigens that are essential for the metastatic process. The transcriptional factor Twist regulates the metastatic ability of 4T1 cells, an experimental mouse model of breast cancer (Yang et al., *Cell* 2004, 117: 927-939). We have utilized this model to probe the ability of IT to elicit antitumor CD8 cytolytic T cells (CTL) that inhibit tumor growth in mice with established metastatic disease. Treatment with local radiation (RT) to the primary tumor and CTLA-4 blockade induced antitumor CD8 T cells inhibiting lung metastases (Demaria et al., *Clin. Cancer Res.* 2005, 11:728-34). The goal of this study was to determine whether Twist is a tumor-associated antigen (TAA) that can be recognized by CD8 T cells.

Methods: H2^d haplotype-restricted CTL epitopes were identified within Twist protein using the SYFPEITHI Epitope Prediction computer-based algorithm. Top-scoring peptides were tested for binding to H2-L^d or H2-K^d in a major histocompatibility complex (MHC) stabilization assay using RMA-S cells expressing the appropriate MHC class I allele. Next, the stability of peptide-MHC complexes was measured by determining the time required for loss of 50% (DC₅₀) of peptide-H2-K^d or H2-L^d complexes at 37°C. Binders were further tested for the ability to sensitize P815 cells to lysis by 4T1 tumor-specific CTL in a standard ⁵¹Cr release assay. γ -interferon production was determined by intracellular cytokine staining. Frequency of peptide-specific CD8 cells was assessed by staining with H2-K^d tetramers.

Results: One out of five candidate peptides bound to H2-L^d, and four out of five candidate peptides bound to H2-K^d. Among them, only peptide 9 (pTw9) formed stable complexes with H2-K^d with a half life of >6 hours on the cell surface. pTw9-specific CTL precursors could be expanded in vitro from mice previously cured of metastatic 4T1 tumors with a combination of local radiation and IT. pTw9-specific CTL showed peptide-specific antitumor effector functions such as killing and γ -interferon production in vitro and recognized Twist+ but not Twist- breast cancer cells. Vaccination experiments indicated that pTw9 peptide is immunogenic in naïve mice. However, variability in response was observed among vaccinated animals. Therefore, optimization of the vaccination is required to assure the consistent development of Twist-specific CTL.

Discussion: Twist regulates mesodermal cell fate during embryonic development. It has been recognized as a key regulator of metastasis in breast cancer and implicated in resistance to Paclitaxel (Cheng et al., *Cancer Res.* 67:1979-87, 2007). Importantly, a clinical study showed that expression of Twist in disseminated tumor cells (DTC) in bone marrow of breast cancer patients correlated with early disease relapse after adjuvant chemotherapy (Watson et al., *Clin. Cancer Res.* 13:5001-9, 2007). Overall, these data suggest that Twist expression by micrometastatic cells enhances their resistance to chemotherapy and is associated with early relapse. IT strategies targeting Twist could be effective as adjuvant treatment in breast cancer patients with Twist+ DTC. We plan to test this hypothesis in the post-surgery 4T1 model. We are applying for additional grants to continue this work.

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P52-7: VACCINATION TARGETING ANTIGENS IDENTIFIED IN TUMOR REJECTION MICE BUT NOT ANTIGENS IDENTIFIED IN TUMOR BEARING MICE HAS TUMOR PROTECTIVE EFFECT

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With a multitude of human tumor antigens identified to date, the challenge lies in identifying those antigens that, when immunologically targeted, would result in tumor rejection. Previously we have shown that neu-transgenic (neu-tg) mice bearing spontaneous tumors have endogenous immune responses against their tumor. A panel of 15 tumor antigens was identified by serological screening of cDNA expression library (SEREX) using sera from tumor bearing mice. Approximately 70% of the tumor antigens identified in neu-tg mice have immunogenic human homologues. We examined the potential therapeutic value of this panel of antigens by vaccinating the mice using plasmids encoding the individual antigens. Unfortunately, none of the tested antigens showed tumor protective effect. We hypothesized that tumor rejection mice may be more suitable than tumor bearing mice in identifying antigens that are associated with tumor rejection. To test this hypothesis, we established two mouse tumor rejection models; (1) parental mice rejecting tumor cell implants; and, (2) tumor regression in neu-tg mice after immunomodulation. Serum samples were collected from mice before and after tumor rejection. Subtractive serological screening of cDNA expression library (SEREX) was used to profile the change in serum antibodies after tumor rejection. After screening 2 cDNA expression libraries, we identified 10 antigens whose antibody responses were preferentially observed in post-rejection sera, including centrosomal protein 290 (Cep290), catenin alpha 1 (Cttna1), FXYD domain containing ion transport regulator 3 (Fxyd3), GPI-anchored membrane protein 1 (GPlap1), heat shock protein 40 (Hsp40), heterogeneous nuclear ribonuclear protein L-like (Hnrpl), mouse mammary tumor virus 1 (Mtv1), talin 1 (Tln1), tumor necrosis factor alpha inducible protein 3 (Tnfaip3), and transmembrane protein 57 (Tmem57). Four of these antigens have immunogenic human homologues. This antigenic repertoire is distinct from the repertoire we previously identified in tumor bearing mice. Preliminary data has shown that vaccination targeting Mtv1 and Fxyd3 has tumor protective effect. Thus, vaccination targeting antigens identified in tumor rejection mice but not antigens identified in tumor bearing mice has tumor protective effect.

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P52-8: ROLE OF INDOLEAMINE 2,3 DIOXYGENASE IN BREAST CANCER METASTASIS FORMATION AND IMMUNOTHERAPY

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Indoleamine 2,3 dioxygenase (IDO) metabolizes tryptophan and was implicated in immunosuppression allowing tumor growth. We hypothesize that IDO expression by tumor cells might result in tryptophan depletion at the tumor-normal tissue interface, causing death of normal cells and thereby increase tumor invasion and metastases formation. To test this hypothesis, we knockdown IDO in highly metastatic IDO+ 4T1 breast tumor cells using siRNA IDO. The isolated siRNA IDO transfected IDO- 4T1 subclone showed a substantial reduction in tumor growth and metastasis formation in comparison to 4T1 parental cells or 4T1 cells transfected with a plasmid containing neo^r and control siRNA sequences. Mice bearing 4T1, 4T1/neo, or 4T1/IDO- tumors developed 66.7, 54.5, and 17.6 pulmonary metastases, respectively. These results indicate that expression of IDO plays a role in metastasis formation. So far our studies failed to confirm that this reduction in local and metastatic growth of IDO- 4T1 cells is a result of increased efficacy of T cell-mediated immunity and probably due to other mechanisms. NT5 breast tumor cells that spontaneously developed in HER-2/neu transgenic mice are highly immunogenic with a low level of IDO gene expression. It was of interest to test whether overexpression of IDO in NT5 cells could suppress immune response and accelerate their growth in FVB/N mice. IDO- NT5-1 clone was transfected with IDO cDNA. IDO+ and IDO- NT5-1 subclones were inoculated into FVB/N mice and their ability to form local tumors and developed lung metastases under current evaluation. Our analysis of in vitro tumor cell proliferation revealed that IDO knockdown in 4T1 tumor cells reduced their rate of proliferation. In contrast, transfection of IDO gene into NT5-1 cells increased their proliferation. These changes in cell proliferation were associated with changes in cell cycle. To further analyze the mechanisms underlying the changes in cell proliferation, microarray analysis of 86 cell cycle genes in IDO+ and IDO- tumor cells was performed. Knockdown of IDO in 4T1 cells resulted in down regulation of 46 genes among which are *Abi1*, *Atm*, *Brca1*, *Brca2*, *Cyclin A2*, *Cyclin B1* and *B2*, *Cyclin C* and *F*, *Cdc25*, *CDK2*, *CDK5rap1*, *Cdkn1b* and *Chk1*. Reduction of *Chk1* (Checkpoint kinase1) gene expression was the most prominent (31-fold). In IDO knockdown cells up-regulation of 14 genes was found. Many of the up-regulated genes are cytoskeleton genes. In NT5-1 cells transfection with IDO gene resulted in up-regulation of 31 genes and only 1 gene *stf* was up-regulated (6.8-fold). In contrast, in IDO knockdown 4T1 cells *stf* was up-regulated by 12-fold. *Stf* is a major

G₂M check point control gene and play an important role in regulation of cell proliferation. Eight genes (*Camk2b*, *Ccnf*, *DST*, *Macf1*, *Mcm4*, *Mki67*, *Ppp3ca*, and *Rad21*) that involved in cell cycle regulation and DNA damage repair were up-regulated in *IDO* transfected NT5-1 cells but the same genes were down-regulated in *IDO* knock-down 4T1 cells. These data indicate a novel biological function of *IDO* related to cell cycle regulation in tumor cells.

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P52-9: EOSINOPHIL GRANULAR PROTEIN COCKTAIL MODULATES THE EXPRESSION OF CANCER GROWTH AND METASTASIS GENES IN MCF-7 MULTICELLULAR SPHEROIDS

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Eosinophils play a significant role in the inflammatory response of allergic reactions as well as helminthic infections. Much of the mechanistic action of these cells is due to the release of toxic granular proteins. We have previously shown that activated eosinophils also inhibit the in vitro growth of the breast cancer cell line MCF-7 and the metastatic cell line MDA-MB-231 in a dose-dependent fashion. Cultured eosinophil supernatants mimicked the activity observed with the eosinophils. Moreover eosinophil cell lines were shown to infiltrate MCF-7 multicellular spheroids and eosinophil granular proteins were observed throughout the spheroid while eosinophils aggregated in the core regions. In addition to toxic granular proteins, eosinophils granules also contain other proteins, among them, perforin and granzyme b, (molecules used by cytotoxic T cells and natural killer cells to kill tumor and viral-infected targets) and also inflammatory and noninflammatory cytokines, IL-4, TNF α , IL-12, that modulate the immune response to cancer as well as have direct anticancer effects. In the present study, we will isolate a panel of these proteins, major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), perforin, granzyme b, IL-4, IL-6, IL-12, and TNF α , from eosinophils cell lines and evaluate their effect on the expression of genes that directly affect tumor growth and metastasis (e.g., erbB2, cyclin D1, cyclin E, p53, E-cadherin, and N-cadherin). Lysates are extracted from eosinophil granules by treatment with 0.01M HCL, purified with the use of amicon ultra centrifugal filter devices, then fractionated on Sephadex G-50. Protein lysates from 21 repetitive HCL extractions revealed a bimodal production of proteins from granules of calcium ionophore-activated eosinophils. There was a high production of proteins in lysates 1-6 followed by low production in lysates 7-14, and then a second surge in production in lysates 15 to 19, while lysates 20 and 21 contained little to no proteins in the panel. On average, 6 of the 10 panel of proteins were present in each lysate (range of 4-8 proteins per lysate). The panel was set using Biorad Experion software and proteins were confirmed by western blot. The predominant proteins in the early and late release included EDN, IL-4, IL-6, TNF α , granzyme B, and MBP. These proteins will be used to develop treatment strategies for the MCF-7 multicellular in vitro model of breast cancer. Eosinophils may play a significant role in immune anticancer activity and offer a tremendous resource of proteins for the potential development of anti-cancer agents.

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P52-10: A NOVEL THERAPEUTIC PARADIGM FOR BREAST CANCER TARGETING TUMOR IMMUNE TOLERANCE WITH SMALL MOLECULE INHIBITORS OF INDOLEAMINE 2,3-DIOXYGENASE

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The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (*IDO*), encoded by the *Ido* gene, is an important component of the complex pathophysiological environment that enables tumors to escape immunosurveillance through the establishment of dominant immune tolerance.¹ Our finding that targeted disruption of the *Bin1* anticancer gene in oncogenically transformed skin keratinocytes resulted in interferon- γ -mediated superinduction of *Ido* gene expression and promoted tumoral immune escape, suggested that this might be a generalizable aspect of tumorigenesis. In particular, we had proposed to test the hypothesis that *IDO* elevation through *Bin1* loss might be an integral stage in the development of breast cancer. Consistent with this notion, frequent attenuation or loss of expression of *Bin1* had previously been reported in malignant breast carcinoma patient biopsies and we had found that the prototypical *IDO* inhibitor, 1-methyl tryptophan (1MT), could act synergistically with conventional cytotoxic chemotherapeutic agents to promote regression of the autochthonous breast tumors that develop in transgenic MMTV-*Neu* mice. However, the results of our studies now suggest to us that the role of *IDO* in breast cancer is more complicated than was originally

predicted. In the mammary gland tumors of MMTV-*Neu* mice, we find no evidence of *IDO* expression or activity, instead, *IDO* is consistently elevated in the tumor draining lymph nodes (TDLNs). This is reflective of the majority of breast cancer patients who were found to have *IDO*⁺ sentinel lymph nodes as well, while only a minority of their tumors were *IDO*⁺. Loss of *IDO* did not have an appreciable impact on primary breast tumor development in several different mouse models whereas in the classical DMBA/TPA skin carcinogenesis model, tumorigenesis was dramatically suppressed by the loss of *IDO*. This likely reflects basic differences in etiology between hormone-driven breast tumors and skin tumors driven by the pro-inflammatory agent TPA. In a mouse model of malignant breast cancer, however, we have found that pulmonary metastases formed less readily and overall survival was significantly extended in an *IDO*-null host, suggesting that, in the context of breast cancer, *IDO* inhibitory compounds may be particularly efficacious against metastatic disease. Mechanism of action studies have led us to identify a novel *IDO*-related gene product, *IDO2*, as the preferential target for the D isomer of 1MT (D-1MT), which recently entered Phase 1 clinical trials. Plans to examine the relative importance of targeting *IDO* versus *IDO2* and the possibility of crosstalk between these two enzymes should have immediate bearing on the current clinical trials with D-1MT.

1 Muller, AM & Scherle, PA. 2006 *Nat. Rev. Cancer* 6:613

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P52-11: THE Pax6 GENE, WHICH IS REGULATED BY TWO PROMOTERS, IS ACTIVATED IN HUMAN BREAST CANCER CELLS

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The CpG island micro-array technique was employed to compare DNA methylation patterns in paired human breast cancer and normal tissues, which led to the discovery that the Pax6 gene could be a possible breast cancer-related cancer-testis antigen. Earlier studies identified Pax6 as an eye development-specific gene. After examining a cDNA panel prepared from 17 human tissues, we found Pax6 had limited expression in testis, the brain, and spinal cord, leading us to believe that it was also a testis- and neural-specific gene. We also became aware that it was activated in many established and primary breast cancer cell lines, as well as breast cancer biopsies. This implied that the Pax6 gene could be a biomarker for this disease. Furthermore, we discovered that two promoters, the adjacent and remote one, governed Pax6. The adjacent promoter's activity was similar in MCF7/Adr, an MDR cell line, and its parental line, MCF7. However, the remote promoter's activity in MCF7/Adr was significantly higher than that in MCF7 cells. These findings suggest that activation of both promoters could be related to breast cancer initiation, while the remote promoter could contribute to cancer progression.

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P52-12: ROLE OF Ets-2 IN TUMOR-ASSOCIATED MACROPHAGES DURING BREAST CANCER PROGRESSION

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While it is known that the most common human tumors are derived from epithelial cells that have undergone multiple genetic alterations, it is also becoming clear that the alterations in the tumor micro-environment are necessary for tumor progression. One such stromal component is the macrophage. Recent studies have shown that deletion of colony stimulating factor-1 (CSF-1), an essential growth factor for growth and differentiation of macrophages, delays pulmonary metastasis in the polyoma middle T antigen breast cancer model in mice. Previous work from our lab has demonstrated that Ets-2 is a nuclear effector of the Ras-Raf-MAP kinase pathway. My hypothesis is that CSF-1 mediates its pro-tumorigenic effects in macrophages via activation of Ets-2.

To test this hypothesis, my project aims to analyze the effects of Ets-2 deletion specifically in the tumor-associated macrophages (TAMs) in the breast tumor microenvironment. To achieve this, I am using a conditional Ets-2 "floxed" allele available in our lab. I am using a non-inducible Lys-Cre transgene to delete Ets-2 specifically in the macrophages. Preliminary results with this system indicate that the gross tumor volume in the experimental animals is similar to that of the controls. Interestingly, the area of the lung lesions is significantly less in the experimental animals as compared to those of the controls. At present I am trying to determine whether it is the exit from the primary tumor site or growth in the lungs that is affected in the experimental animals.

Microarray and real-time PCR analysis of mammary TAMs indicate that anti-angiogenic factors may be downregulated in the Ets-2 deleted TAMs.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0735.

TARGETED THERAPIES II

Poster Session P53

P53-1: BLOCKAGE OF CYR61- α V β 3 INTEGRIN INTERACTION RESTORES TAXANE-SENSITIVITY IN TRIPLE NEGATIVE BREAST CANCER CELLS

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The angiogenic factor Cyr61 (also known as CCN1) plays a key role in both the maintenance and enhancement of a malignant phenotype in breast cancer. Cyr61 is overexpressed in about 30 percent of triple negative breast carcinomas, whereas Cyr61 expression levels in normal breast tissues are negligible. Cyr61 expression is highly correlated with advanced disease. Although further studies are needed to definitively establish Cyr61 as a prognostic marker, it has been demonstrated that Cyr61 overexpression is significantly associated with tumor stage, tumor size, lymph node, and age. We recently demonstrated that Cyr61 overexpression renders human breast cancer cells highly resistant to the microtubule-interfering agent paclitaxel (Taxol), the drug of choice for the treatment of metastatic breast cancer. We confirmed that expression of α v β 3, a Cyr61 receptor, is markedly up-regulated in breast cancer cells expressing Cyr61. Our most recent data demonstrate that the functional blockade of 3 α v β 3 with a synthetic chemical peptidomimetic, based upon the α v β 3 RGD (Arg-Gly-Asp) motif, is specifically cytotoxic toward Cyr61-overexpressing breast cancer. Pharmacological interference with the Cyr61/ α v β 3 interaction restores Taxol efficacy, implying that a previously unrecognized Cyr61/ α v β 3-driven cellular signaling actively modulates breast cancer cell growth, apoptosis, and chemosensitivity. That is, Cyr61/ α v β 3-driven cellular signaling regulates breast cancer survival and growth and promotes chemoresistance as it enhances aggressiveness. To verify that the sole interaction between Cyr61/ α v β 3 integrin promotes chemoresistance, namely Taxane-resistance, we took advantage of a Cyr61 mutant with a point mutation at the position 125 of the protein (D125A) and which lacks the ability to interact with α v β 3. The studies showed that while Cyr61 induced the Taxol resistance of breast cancer cells, the molecular interference between Cyr61/ α v β 3 using the D125A mutant, resulted in Taxol sensitivity, concluding that indeed Cyr61/ α v β 3 interaction plays a pivotal role in Taxol resistance. Consequently, it seems plausible that Cyr61 expression may determine the subset of triple negative breast cancer patients who are likely to progress and are likely to become refractory to Taxane-based therapies. Our studies demonstrate that expression of Cyr61 induces the expression of α v β 3 in the epithelial compartment. Nevertheless, it is also known that α v β 3 is highly expressed in the endothelial compartment and a marker of angiogenesis. Since Cyr61 is a secreted protein, we can envision that the identification of a Cyr61/ α v β 3 autocrine loop suggests that targeting Cyr61/ α v β 3 may simultaneously prevent breast cancer tumorigenesis, angiogenesis, and chemoresistance. Our findings open a novel molecular avenue in the management of metastatic breast cancer but will also reveal a novel predictive marker for treatment responses in breast cancer. These studies provide a rationale and proof-of-concept to develop clinical trials based upon Cyr61/ α v β 3 targeted therapies alone or in combination with Taxane-based therapies.

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P53-2: FUNCTIONAL PROTEOMICS AND COMPUTATIONAL MODELING OF THE SIGNALING NETWORK TO IDENTIFY TARGETS FOR THERAPY

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Background: Small molecule inhibitors targeting signaling molecules are rapidly being developed and tested in clinical trial. However, when used as single agents, they have generally been disappointing. There could be a number of reasons for failure of these drugs. One of the reasons could be at the signaling network level. The signaling network in the cell is complex and contains numerous homeostatic feedback loops. Unexpected disruption of these loops by targeted therapeutics could lead to unpredictable and unfavorable outcomes. Understanding the effects of the targeted inhibitors on the signaling network and rational identification of targets may greatly improve how we use these inhibitors.

Brief Description of Methodologies: A functional proteomic analysis coupled to computational modeling of the signaling network in response to targeted inhibitors was used. A panel of breast tumor cells (MDA231, BT549, MCF7, MDA468, T47D, SKBr3, and nontransformed MCF10A) were treated with inhibitors to MEK, AKT, and EGFR. The cells were also treated with EGF and controls included vehicle treatment. The cell lysates were spotted onto reverse phase protein arrays and the expression levels and phosphorylation levels of 45 signaling proteins were determined. We developed a computational network model called PathwayOracle to visualize the network. We also developed a signaling petri-net simulator to model how the inhibitors and EGF were altering signal flow within the network.

Results to Date: Using our systems biology approach, we determined that that MEK inhibitors increased AKT phosphorylation in a panel of breast epithelial cells. We determined the mechanism leading to the increase in AKT and elucidated two feedback loops. Rational therapeutic combinations targeting MEK and components of the feedback loops reversed the increase in AKT phosphorylation, its downstream signaling, and proliferation.

Conclusion and Impact on Breast Cancer Treatment: A systems understanding of the signaling network identified targets for therapy. Combinatorial targeting of the signaling network resulted in a significant functional decrease in cell proliferation providing rational therapeutic combinations for clinical development. Approaches to identify and overcome inadvertent changes in signaling by small molecule inhibitors will be required to determine optimal combinations in a preclinical setting facilitating their evaluation in clinical trials.

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P53-3: DELIVERY IN VITRO AND IN VIVO IN MICE OF AN ANTISENSE MORF USING HERCEPTIN AS CARRIER

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Background and Objectives: We intend to use phosphorodiamidate morpholino (MORF) antisense oligomers in breast cancer treatment. In part because of its neutral charge, antisense MORF oligomers require transmembrane carriers such as TAT or polyarginine for delivery into breast and other tumors. We have now investigated the internalizing antitumor antibody Herceptin for this purpose. In this study, streptavidin was again used as a convenient link between the biontynylated antibody and antisense MORF. This delivery nanoparticle was studied in tumor cells expressing the target antigen and target mRNA both in vitro and in vivo.

Methods: The 25-mer antisense MORF oligomer against the RhoC mRNA and its sense control were purchased with either a biotin or a lissamine on the 3'-end and a primary amine on the 5'-end to conjugate either with NHS-biotin (fluorescent MORF) or NHS-MAG3 (biotin MORF) for radiolabeling with ^{99m}Tc. The delivery nanoparticles were formed by mixing the fluorescence labeled or radiolabeled biotinylated MORF with streptavidin followed by addition of biotinylated Herceptin at a 1:1:1 molar ratio. Both the inflammatory breast cancer cells SUM190 (Her2+, RhoC+) and control SUM149 (HER2-, RhoC-) were incubated with radiolabeled or fluorescence labeled nanoparticle at 10 nM for various times. Cell accumulations were measured by counting ^{99m}Tc and subcellular distribution was determined by confocal fluorescence microscopy. The fate of the nanoparticle in the cytoplasm was also investigated by size exclusion HPLC. Nude mice bearing either xenografts were injected IV with nanoparticles containing 1.8 μ g of antisense MORF and were sacrificed at 6, 12, or 24 h. Tumor slices were viewed by fluorescence microscopy using tumors from mice receiving the nanoparticle without lissamine to correct for tissue background. The ability of the antisense MORF nanoparticle to inhibit RhoC when incubated at 100 nM was evaluated in SUM190 cells by the Boyden Chamber invasion assay.

Results: The accumulation in culture of both nanoparticles in SUM190 cells was 20-40 times that of SUM149 cells and the MORF distributed uniformly throughout the cytoplasm and without evidence of entrapment. HPLC analysis of the cytoplasm at 6 to 8 h incubation showed that a fraction of labeled MORF was released from the nanoparticle. Microscopy examination of tumor slices shows that the MORF was delivered only into the SUM190 cells. Incubation with the antisense nanoparticle at 100 nM reduced the migration of SUM190 cells by about 90%.

Conclusions: Internalizing antitumor antibody Herceptin can be used as an efficient carrier to specifically deliver antisense MORFs into HER2 expressing breast tumor cells in vitro and in vivo. The results of this study provided additional evidence that the antisense oligomer is released within cells incubated with nanoparticles and appeared to be unhindered in targeting its mRNA.

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P53-4: TARGETED LIPOSOMAL DELIVERY OF ALPHA-PARTICLE EMITTER, ACTINIUM-225

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Alpha particle-emitting radionuclides hold great promise as therapeutic agents for cancer. Astatine-211 (At-211) and bismuth-213 (Bi-213) have been investigated clinically. Both have short half-lives (7 h and 46 min, respectively) and are appropriate for rapid targeting situations. In late-stage breast cancer patients (with measurable liver or bone metastases), long-lived alpha particle emitters are required to reach distant metastases that have developed their own vasculature. Ac-225 has a 10-day half-life and daughters that yield 4 alphas. This radionuclide is 1,000-fold more effective than Bi-213; however, it has also proved to be far more toxic. The increased efficacy and toxicity are a result of the alpha-particle emitting intermediates. When these are confined to the target cells, efficacy is increased; when they distribute throughout the body, toxicity is increased. This is a fundamental difficulty for antibody-mediated targeting since the bond holding the Ac-225 atom to the antibody will be broken after decay of Ac-225.

This will leave the first daughter in the decay chain free to distribute throughout the body where it will decay and subsequently yield additional alpha emissions to normal organs from subsequent daughter decays. We investigated the feasibility of using liposomal vesicles to encapsulate and retain Ac-225 and its radioactive daughters for delivery to tumor sites. Our results have shown that there is a fundamental limitation to this approach because of the 100 nm recoil distance of daughter nuclei following alpha-particle emission (1). This limitation requires larger liposomes for adequate retention of daughters (2). The requirement for larger liposomes limits application of this approach to locoregional administrations. We are, therefore, considering application of this approach to ovarian carcinoma therapy by intraperitoneal administration.

1. Sofou S, Thomas JL, Lin H, McDevitt MR, Scheinberg DA, Sgouros G. Engineered liposomes for potential α -particle therapy of metastatic cancer. *J. Nucl. Med.* 2004; 45:253-60.
2. Sofou S, Kappel BJ, Jaggi JS, McDevitt MR, Scheinberg DA, Sgouros G. Enhanced retention of the alpha-particle-emitting daughters of Actinium-225 by liposome carriers. *Bioconjug. Chem.* 2007; 18(6):2061-7.

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P53-5: SIGNIFICANCE OF CHEMOTHERAPY-INDUCED RECEPTOR TYROSINE KINASE ACTIVATION AND DEGRADATION IN BREAST CANCER

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Background and Objectives: Our preclinical data indicate that: (1) cisplatin, gemcitabine, and doxorubicin treatment of breast cancer cells could cause EGFR and ErbB2 phosphorylation that facilitates the receptor ubiquitination and subsequent degradation, and (2) drug-induced receptor degradation is associated with cytotoxicity and radiochemosensitization. The main objective of this research is to understand the mechanism and significance of initial EGFR and ErbB2 phosphorylation and its subsequent degradation in cell death and radiochemosensitization in preclinical models.

Methods: To understand the significance of drug-induced initial phosphorylation and degradation, we have synthesized and expressed full-length wild-type (WT) EGFR in EGFR negative Chinese hamster ovary cells (CHO). We are in the process of creating a Y1045F EGFR point mutation that will be used to produce stable Y1045F EGFR-CHO clones. We expect expression of this clone to inhibit drug-induced receptor degradation and ultimately to antagonize drug-induced toxicity as well as chemo and radiosensitization. We are working on two other approaches to either block or promote receptor degradation: (1) Expressing c-cbl mutant lacking the ring finger domain that lacks the ubiquitin ligase function and (2) inhibiting HSP90 activity using its specific inhibitor geldanamycin. We have already synthesized WT and mutant c-cbl and are validating these constructs before we express them in the CHO clones that express WT and Y1045F EGFR. This approach will permit us to block EGFR degradation independent of receptor phosphorylation. These CHO clones will be utilized to test our hypothesis that EGFR activation-mediated degradation plays a critical role in drug-mediated chemo- and radiosensitization. Immunoblotting and clonogenic assays will be employed to validate the newly developed system. The effect of inhibition of HSP90 on chemo- and radiosensitivity will be assessed by clonogenic assay.

Results: Preliminary experiments performed in EGFR-expressing CHO cells have shown that cisplatin induces initial EGFR phosphorylation leading to its degradation. Our second hypothesis is that exposure to chemotherapy (e.g., cisplatin) induces a stress response that increases levels of HSP90, which inhibits EGFR/Her-2 degradation. If this were true, then blocking HSP90 activity by its specific inhibitor geldanamycin would sensitize breast cancer cells to radio and chemotherapy. In fact, our preliminary data indicate that pretreatment with geldanamycin sensitizes MDA-MB-468 cells to both chemo and radiation therapy. We also found that conditions that sensitize MDA-MB-468 cells to chemo and radiotherapy correlate with enhanced receptor degradation.

Conclusions: Ectopically expressed EGFR in CHO cells is activated and degraded by cisplatin in a manner similar to that seen in breast cancer cell lines. Thus we feel this system can be used to identify the mechanism of receptor activation-degradation produced by treatment with chemotherapy. We have also found that inhibition of HSP90 activity promotes both ErbB2 and EGFR degradation and sensitizes cells to cisplatin and to radiation, indicating the significance of receptor degradation in chemo and radiosensitization.

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P53-6: STUDY OF THE ROLE OF DIFFERENT DOWNSTREAM MEDIATORS OF THE TGF β -1 SIGNALING PATHWAY IN THE REGULATION OF FACTORS AFFECTING BREAST CANCER-ASSOCIATED OSTEOLYTIC BONE METASTASIS

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Background and Objectives: The TGF β 1 signaling pathway and its downstream mediators play a primary role in the establishment of bone destruction during metastasis of breast cancer to the bone site. The goal of this study is to delineate the TGF β 1 signaling pathway mediating the production of osteolytic factor PTHrP (parathyroid hormone-related protein) and various pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, and IL-11.

Methods: Human breast cancer cell lines of various metastatic potentials, including MDA-MB-231 (osteolytic, but low-level metastasis), bone-seeking MDA-MB-231(hm) (highly metastatic), MDA-MB-468 (low metastasis), and MCF-7 (osteoblastic), were evaluated. The total levels of SMAD2/3 and TGF β 1 dependent SMAD2 and SMAD3 phosphorylation were determined by western blots. Nuclear translocation of activated forms of SMAD2 and SMAD3 was determined by microscopy coupled with immunofluorescent staining. The IL-6, IL-8, and IL-11 levels were determined by ELISA. PTHrP levels were examined by real-time PCR. Expression of TGF β receptors RI and RII were determined by RT-PCR. RNA silencing technology and overexpression of dominant-negative isoform of p38 MAPK were used to evaluate the role of R-SMADs and p38 MAPK in breast cancer cells.

Results: We have shown the upregulation of IL-6, IL-8, and IL-11 cytokines and PTHrP in breast cancer cell lines in response to TGF β 1. The upregulation of these osteolytic factors is mediated by the rapid activation of p38 MAPK and R-SMADs, specifically SMAD2 and SMAD3 through the TGF β 1/TGF β R system. However, the four breast cancer cell lines, MCF-7, MDA-MB-231, MDA-MB-231(hm), and MDA-MB-468, show differential expression and upregulation of these osteolytic factors in response to TGF β 1. As compared to the parental cell line MDA-MB-231, MDA-MB-231(hm) cells express four-fold higher basal levels of IL-11 (0.44 ng/mL) and threefold lower basal levels of both IL-6 (0.27 ng/mL) and IL-8 (0.24 ng/mL) cytokines. The MCF-7 cell line with little or no TGF β RII shows no significant upregulation of PTHrP nor expresses any of the cytokines. In comparison, MDA-MB-468 cells show elevated expression of only IL-8 (0.2 ng/mL) and PTHrP in response to TGF β 1. The production of cytokines and upregulation of PTHrP are not dependent on the SMAD4-dependent nuclear translocation of activated SMAD2 and SMAD3 since this translocation is observed in all the breast tumor cell lines used here except MCF-7. Thus, the study of induction of TGF β 1-responsive osteolytic factors in these four cell lines offers a unique genetic background to study this pathway.

Conclusion and Impact on Breast Cancer Research and Treatment: The results presented here imply that the consequence of TGF β 1 on osteolytic bone destruction depends upon several factors including the status of TGF β receptors, status of SMAD4 levels and the capacity of TGF β 1 to induce both SMAD and MAPK pathways, PTHrP, and IL-11 expression. This crucial understanding of the mechanism of TGF β 1 signaling can pave a way for the discovery of new drugs designed to alleviate complications arising from breast cancer-associated osteolytic bone destruction or better yet prevent breast cancer-associated bone metastasis altogether.

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P53-7: TARGETED CHARGE-REVERSAL DRUG CARRIERS FOR NUCLEAR DRUG DELIVERY FOR BREAST CANCER CHEMOTHERAPY

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Most cancer chemotherapy drugs target nuclear DNA to cause DNA damages and/or topoisomerase inhibition to induce cell death (apoptosis). In addition to the membrane-associated multidrug resistance, drug-resistant cancer cells also have many intracellular drug-resistance mechanisms to limit the access of drugs to the nucleus. Consequently, only a small percentage of drugs delivered into the cytosol finally reach the nucleus in drug-resistant cells. Thus, a drug carrier capable of localizing and releasing drugs directly into the nucleus would circumvent both the multidrug resistance and intracellular drug resistance mechanisms, leading to a high therapeutic efficacy.

Cationic polymers such as polyethyleneimine (PEI) and polylysine (PLL) can carry DNA across the cell membrane and harness the molecular motors to enter the nucleus. However, PEI and PLL have a rapid plasma clearance due to their positive charges from their amine groups.

An ideal regime would be to activate the cationic charges only in cancerous tissues or their intracellular compartments. Herein, we report drug carriers with a negative-to-positive charge reversal triggered by the solid tumor extracellular (pH <7) or lysosomal (4–5) acidity for nuclear drug delivery. The carriers are negatively charged in the bloodstream and have long circulation times. The drugs loaded in the charge-reversal carriers have a higher cytotoxicity than free drugs.

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P53-8: ASSESSMENT OF FIRST GENERATION NANOGELS FOR TARGETED siRNA DELIVERY TO TUMOR VASCULATURE

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The inhibition of tumor angiogenesis has significant potential as a therapeutic modality in the treatment of breast cancer. Targeting the delivery of small, interfering RNA (siRNA) to activated breast microvascular endothelial cells (MVEC) to decrease the expression of proteins critical to breast MVEC during tumor angiogenesis could lead to less toxic and more effective breast cancer treatments but is limited by the absence of efficient targeted drug delivery vehicles.

The ability of nanoparticles composed of carbamate cross-linked polyethylene glycol (PEG)/polyethylenimine homopolymers (Nanogels/NGs) to target siRNA delivery to activated breast MVEC was assessed. A peptide (V1) reported to have high affinity for VEGFR2, a protein upregulated on the surface of activated MVEC, was conjugated to 9:1 (PEG:PEI) NGs by a 3.4 kDa MAL-PEG-NHS crosslinker (NG-V1). NGs were modified with V1 at 10, 15, or 20% of NG NH₂ groups / mg NG (~2.3 μmoles NH₂ groups / mg NG). Appropriate levels of peptide substitution were confirmed for each NG-V1 construct by the Sakaguchi assay.

NG-V1 complexation of model siRNA (dsDNA) as a function of N/P ratio was assessed up to N/P 20 by agarose gel. NG-siRNA complexes migrated distances that decreased with increasing N/P, indicating that electronegative NG-siRNA complexes were formed over this range of N/P ratios. NG-V1 protection of dsDNA as a function of N/P ratio was assessed by a fluorescence-based assay comparing levels of dsDNA within NG-siRNA complexes alone or treated with DNase. NG-V1 (10% or 15%) protected dsDNA against DNase activity to a similar extent as unmodified NGs at N/P ratios > 8/1. NG-V1-20 was discarded due to its relative inability to protect dsDNA.

Breast MVEC (target cells) were isolated from the Immortomouse strain by FACS, double selecting for the TNF-α-induced expression of V-CAM and E-Selectin. Transfection activity of NG-V1-siRNA at the lowest N/P ratio that protects ≥ 95% model siRNA (15/1) was assessed in Immortomouse breast MVEC using siRNA against Kif11 (Eg5), a protein whose reduction causes mitotic arrest. Breast MVEC were transfected with SMARTpool Kif11 siRNA or non-targeting SMARTpool siRNA as a control. A slight increase in growth inhibition by Kif11 siRNA over control siRNA was observed for NG alone or NG-V1-10 that was not observed at the mRNA level by Q-RT-PCR. In general, an increase in V1 modification resulted in a decrease in breast MVEC growth. Levels of a metabolic indicator (CAM) correlated to the total number of cells (CyQUANT), suggesting that growth inhibition was not associated with cytotoxicity. These results indicate that NG and NG-V1 constructs inhibit breast MVEC growth in vitro irrespective of loaded siRNA by some mechanism unrelated to detectable cytotoxicity.

In conclusion, first generation NGs having biostable carbamate cross-links were found to be insufficient for siRNA delivery to breast MVEC. Given that similar observations with delivering nucleoside analogs were overcome with biodegradable NGs, we are now assessing the potential of biodegradable NGs for targeted siRNA delivery. V1 was also recently shown to have affinity for purified VEGFR2 cofactor, NRP1, and not purified VEGFR2. Therefore, a high affinity peptide (K237) identified by phage display against the interaction of VEGF with VEGFR2 is being used in place of V1.

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P53-9: SELECTIVE ONCOLYTIC THERAPY FOR HYPOXIC BREAST CANCER CELLS

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Hypoxic breast cancer cells are more refractory to conventional therapy compared to normoxic cells. Oncolytic viruses, such as the R3616 derivative of herpes simplex I (HSV-I), are now used in clinical trials. We tested whether hypoxia-mediated ERK (extracellular receptor kinase) activation renders cancer cells more permissive to R3616, which is defective in blocking the host's inhibition of viral protein synthesis. We observed a sevenfold increase in plaque forming units (pfu) when hypoxic MDA-MB-231 cells were inoculated with R3616, compared to normoxic cells. Indeed,

hypoxic MDA-MB-231 cells were only slightly less permissive than hypoxic Vero cells. We observed ample HSV-I gC (envelope protein) expression in hypoxic MDA-MB-231 cells after 24 hrs of infection. Although hypoxic MCF-7 cells were threefold more permissive than normoxic cells, overall titers were very low (< 100 pfu). Since basal ERK1/2 phosphorylation is high in MDA-MB-231 normoxic cells, other signaling pathways may render MDA-MB-231 hypoxic cells permissive to R3616 and will be the focus of future studies. Thus, hypoxia may render particular cancer cells more permissive to HSV-I derived viruses.

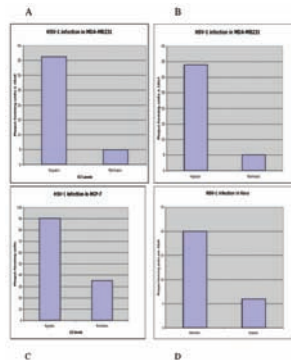


Figure 1. Hypoxic MDA-MB-231 cells are more permissive to HSV-1-derived virus

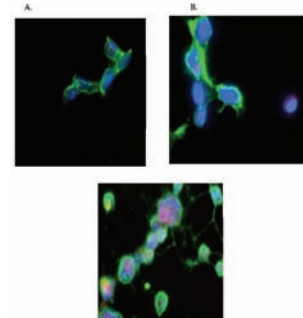


Figure 2. HSV-1 envelope protein is abundantly produced in hypoxic MDA-MB-231 cells

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P53-10: ANTITUMOR EFFECTS OF A NOVEL ANTI-HERV-K MONOCLONAL ANTIBODY IN BREAST CANCER

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Background and Objectives: Recent evidence indicates that human cancer cells re-activate the expression of latent human endogenous retroviral (HERV) proteins. Our recent results demonstrate that HERV-K env proteins are widely expressed in human breast cancer (BC) biopsies. These novel antigens for BC elicit both serological and cell-mediated immune responses against HERV-K protein. Our hypothesis is that these endogenous viral antigens can be used as targets for therapeutic use against BC and other solid tumors where HERV proteins have also been found to be expressed. This hypothesis is based on the prevalence of HERV-K expression in human BC and the immunogenicity of HERV-K protein products in patients. In this study, monoclonal and single-chain antibodies against HERV-K were generated in our laboratory and tested for antitumor effects in vitro and in vivo. Here we report for the first time that a novel anti-HERV-K env antibody can be used for the development of passive immunotherapy against BC.

Methodologies: HERV-K env surface fusion protein and its monoclonal antibody, 6H5, were affinity purified using an AKTA FPLC system. ELISA and western blot screening revealed that 6H5 had the highest specificity and sensitivity toward HERV-K. Surface expression of HERV-K env protein in cell lines was determined by confocal microscopy, dry cell ELISA, western blot, and flow cytometry using 6H5. MTT cell proliferation assay was employed to measure cellular proliferation, and crystal violet staining was used to determine in vitro cytotoxicity of 6H5. An annexin V apoptosis assay was used to evaluate apoptosis induced by 6H5. SCID female mice bearing human breast tumors were used to test antitumor effects of 6H5 in vivo. Tumor growth was monitored, and histology was evaluated among groups at the end of the tumorigenesis study. The TUNEL assay defined cells as apoptotic if nuclear localized brown staining was observed.

Results to Date: Cell surface expression was detected in 83% of MDA-MB-231 and 56% of MCF-7 breast cancer cells but not in the untransformed MCF-10A (5%) or the ras-transformed breast cell line MCF-10AT (11%). Surface expression was also detected in ZR-75-1, T47D, MDA-MB-453, MDA-MB-435 eB1, and BT474 breast cancer cell lines. The env protein was glycosylated in these cancer cell lines. 6H5 was able to inhibit HERV-K⁺ breast cancer cell proliferation and induce breast cancer cells to undergo apoptosis in vitro. Very little or no cytotoxicity of 6H5 was observed toward MCF-10A and MCF-10AT cells. 6H5 induced greater percentages of apoptosis in MDA-MB-231 (53%), MCF-7 (41%), T47D (29%), and MDA-MB-453 (48%) than in MCF-10A (10%) and MCF-10AT (8%) cells. Increased apoptosis was correlated with the surface expression of HERV-K env protein. Tumor sizes were significantly reduced, and onset of tumorigenesis was significantly delayed in 6H5-treated immunodeficient mice bearing MDA-MB-231 and MCF-7 tumors. Antibodies induced cancer cells to undergo apoptosis, as assessed by TUNEL assay, in tumors whose onset was delayed.

Conclusion: HERV-K env protein is a unique anticancer target, and 6H5 anti-HERV-K monoclonal antibody could be of therapeutic value for HERV-K-positive human cancers. These antibodies against HERV-K env protein may be useful for immunotherapy against HERV-K-positive cancers.

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P53-11: SMALL MOLECULE INHIBITORS OF NF- κ B AS A NOVEL TREATMENT MODALITY FOR BREAST CANCER

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NF- κ B, a transcription factor that commonly exists as a heterodimer of a p50 and p65 subunit, contributes to cell proliferation and the invasion of cancer cells. NF- κ B is constitutively active in many ER-negative breast cancer cell lines and breast tumors so a novel approach to inhibit the activity of NF- κ B may have therapeutic potential against this disease.

The transcriptional activity of the p65 subunit of NF- κ B is regulated by phosphorylation. Phosphorylation of serine 276 leads to the interaction of p65 with transcriptional coregulators, including CBP. Mutation of serine 276 downregulates the expression of genes involved in breast cancer metastasis, including intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), and genes involved in angiogenesis, including cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8). It is thus hypothesized that inhibition of serine 276 phosphorylation may prevent breast cancer metastasis and angiogenesis.

A structural pocket near serine 276 was identified in the crystal structure of p65. It was hypothesized that a small molecule that specifically binds in this cleft would inhibit serine 276 phosphorylation and potentially prevent the interaction of p65 with transcriptional coregulators. Using *in silico* molecular docking, a structure-based approach for the identification of lead compounds, small molecules that have the potential to bind to a structural pocket near serine 276 of p65 were identified. Approximately 220,000 compounds available from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) were screened. Each small molecule was positioned in the selected site in 1,000 different orientations, and the best orientations and their scores (contact and electrostatic) were calculated. The top 20 compounds were obtained and tested for inhibition of constitutive serine 276 phosphorylation in 231 breast carcinoma cells. Two compounds, designated compound No. 5 and compound No. 7, were found to potentially inhibit serine 276 phosphorylation in breast cancer cell lines (Figure 1).

The effect of compounds No. 5 and No. 7 on the expression of ICAM-1, VCAM-1, IL-6, and IL-8 is currently being tested. It is anticipated that these small molecules will inhibit breast cancer metastasis, and it is hoped that these compounds will be further modified and optimized for the treatment of breast cancer in the future.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0633.

P53-12: DEVELOPMENT OF HYPERSTABLE MICELLES AS CARRIERS FOR SPARINGLY SOLUBLE ANTICANCER DRUGS

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Most anticancer agents are characterized by poor solubility and high hydrophobicity. This is especially true for anticancer drugs currently used in the battle against breast cancer. Paclitaxel (Taxol®) is an extremely effective drug used against breast cancer. However, because of its low solubility, paclitaxel is currently either dissolved in a toxic solvent that is then diluted with the appropriate buffer to yield a suspension that is administered intravenously to the patient or it is administered as a suspension of paclitaxel protein-bound particles. This latter formulation does not use toxic solvents, but it can cause severe allergic reactions. Both formulations show how important it is to find a proper and effective carrier that will not elicit toxic and/or allergic responses. To this purpose, we have designed and synthesized novel hyperstable micellar carriers composed of semifluorinated triblock copolymers (Figure 1). The use of micelles in drug delivery is particularly indicated for low-solubility, highly hydrophobic drugs like paclitaxel, for drugs that are not very stable under physiological conditions, and last, but not least, as a tool to direct delivery to specific tissues or organs. Micelle stability is an important factor in micellar drug delivery. In fact, upon intravenous delivery, most micelles experience conditions of extreme dilution and consequently dissociate, freeing their precious cargo. Our approach to hyperstable micelles is based on the coupling of a

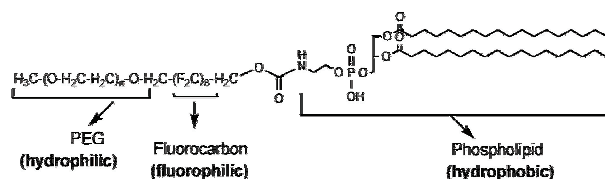


Figure 1. Molecular structure of the synthesized tri-block copolymer

hyper-hydrophobic perfluorinated chain with the hydrophilic and hydrophobic segment of the polymer. In our design, the hydrophilic poly(ethyl glycol) block ensures water solubility. The intermediate fluorocarbon block adds extra stability to the aggregates formed in water. The phospholipid block allows efficient binding and encapsulation of paclitaxel. Furthermore, the stability effect associated with the formation of an intermediate fluorocarbon phase also allows protection of the encapsulated pharmaceutical as the drug will be sealed from the external environment by an energetically stable fluorocarbon phase. This design allows sustained release of very toxic drugs and compounds that are sensitive to their immediate environment. It also allows the micelles to circulate in the blood stream long enough to reach the site of tumors by simply diffusing through the leaky blood vessels that invariably surround tumors. The mean diameter of the micelles formed by the new copolymer was measured to be 15 ± 3 nm. The critical micelle concentration (CMC) was determined to be 0.65 millimolar.

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P53-13: INACTIVATION OF NF- κ B BY 3, 3-DIINDOLYLMETHANE (DIM) CONTRIBUTES TO INCREASED APOPTOSIS INDUCED BY CHEMOTHERAPEUTIC AGENT IN BREAST CANCER CELLS

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Constitutive activation of Akt or NF- κ B has been reported to play a role in de novo resistance of cancer cells to chemotherapeutic agents, which is a major cause of treatment failure in cancer chemotherapy. Previous studies have shown that 3, 3'-diindolylmethane (DIM), a major *in vivo* acid-catalyzed condensation product of indole-3-carbinol (I3C), is a potent inducer of apoptosis, inhibitor of tumor angiogenesis, and inactivator of Akt/NF- κ B signaling in breast cancer cells. However, little is known regarding the inactivation of Akt/NF- κ B that leads to chemosensitization of breast cancer cells to chemotherapeutic agents such as Taxotere®. Therefore, we examined whether the inactivation Akt/NF- κ B signaling caused by B-DIM could sensitize breast cancer cells to chemotherapeutic agents both *in vitro* as well as *in vivo*. MDA-MB-231 cells were simultaneously treated with 15 to 45 μ M B-DIM and 0.5 to 1.0 nM Taxotere for 24 to 72 hours. Cell growth inhibition assay, apoptosis assay, EMSA, and western blotting were performed. The combination treatment of 30 μ M B-DIM with 1.0 nM Taxotere elicited significantly greater inhibition of cell growth compared with either agent alone. The combination treatment induced greater apoptosis in MDA-MB-231 cells compared with single agents. Moreover, we found that NF- κ B activity was significantly decreased in cells treated with B-DIM and Taxotere. We also have tested our hypothesis using transfection studies followed by combination treatment with B-DIM/Taxotere and found that combination treatment significantly inhibited cell growth and induced apoptosis in MDA-MB-231 breast cancer cells mediated by the inactivation of NF- κ B, a specific target *in vitro* and *in vivo*. These results were also supported by animal experiments that clearly showed that B-DIM sensitized the breast tumors to Taxotere, which resulted in greater antitumor activity mediated by the inhibition of Akt and NF- κ B. Collectively, our results clearly suggest that inhibition of Akt/NF- κ B signaling by B-DIM leads to chemosensitization of breast cancer cells to Taxotere, which may contribute to increased growth inhibition and apoptosis in breast cancer cells. The data obtained from our studies could be a novel breakthrough in cancer therapeutics by using nontoxic agents such as B-DIM in combination with other conventional therapeutic agents such as Taxotere.

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P53-14: NOVEL IN SITU GEL DRUG DELIVERY SYSTEM FOR BREAST CANCER TREATMENT

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Background and Objectives: MUC1 is a tumor-associated transmembrane glycoprotein that is overexpressed (>10-fold) in 90% of breast carcinomas and is being used as a target for active and passive cancer immunotherapy in many clinical trials. Paclitaxel (PTX) has been used against a wide variety of tumors including breast cancer. However, poor aqueous solubility, poor bioavailability, and toxicities have been major obstacles for PTX. Therefore, the major objective of these studies was to utilize the overexpression of mucin associated with cancerous breast tissues as a targeting strategy

to develop a novel in situ gel delivery system for the safe, effective, and sustained release of PTX. This system consists of a natural ionic polymer (chitosan) and a mucoadhesive, glycerylmonooleate (GMO) at slightly acidic conditions. However, when this viscous solution is injected locally close to tumors at the biological pH, the ionic polymer deprotonates and forms a biodegradable gel that sustains the delivery of PTX.

Methodology: (1) The optimal formulation conditions include dispersion of a known amount of paclitaxel in the citric acid solution followed by the addition of chitosan (3% w/v), which was finally mixed with the melted GMO with sonication. An HPLC method was developed and validated to determine the in vitro release of PTX from the gel, the in vitro cellular uptake and MTT cytotoxicity of the delivery system in MDA-MB-231 breast cancer cells. (2) The safety and effectiveness of a localized in situ gel to the systemic delivery of PTX were compared and evaluated using cancer xenograph models of Clone-66 murine breast cancer cells in Balb/c mice and human breast cancer cell lines, MDA-231 and MDA-453, in immunodeficient SCID mice. These mice received either a locally injected in situ gel or the same dose of PTX systemically. The rate of tumor growth was monitored.

Results to Date: In the initial phase of this project, the in situ gel was formulated and extensively characterized. However, the low pH of the in situ gel and homogeneous incorporation of PTX into the gel became considerable biological challenges. Therefore, a nanoparticle (NP) approach was explored to circumvent these challenges. This new approach provided conceptual proof that chitosan/GMO can form polycationic NPs (400 to 700 nm) with sustained PTX release. The NPs show evidence of mucoadhesive properties; a 4-fold increased in cellular uptake and a 1000-fold decrease in the IC₅₀ of PTX in MDA-MB-231 cells. Tumor development in vivo was visible after 6 days and measurable on day 9 after the initial MDA-MB-231 cell injection. The tumor diameter increased at a constant rate for all the groups between days 7-14. After a single intratumoral bolus dose of the PTX formulated NPs, a significant decrease (100%) in tumor diameter was observed on day 15 and at day 18 the tumor diameter reached a 4-fold maximal decrease in both the mammary pad and the flank when compared to control, placebo, or intravenous PTX administration. The difference was reduced to approximately 2-fold by day 21 when compared to control, placebo, and intravenous PTX administration.

Conclusions: The advantages of this novel drug delivery system allow lower doses of PTX to achieve a significantly increased therapeutic effect, thus presumably minimizing the adverse side effects. The therapeutic efficacy of NPs via intravenous administration is currently under investigation.

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P53-15: SYNTHESIS OF RASPBERRY-LIKE CORE-SHELL NANOPARTICLES FOR ANTITUMOR DRUG DELIVERY

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A versatile strategy based on noncovalent interactions of biological particles and polymers has been developed to obtain raspberry-like core-shell nanoparticles (Figure). The structures have very good coverage of the bioparticles on the surface of polymeric spheres as characterized by TEM and SEM analyses. Hydrophobic drugs can be efficiently encapsulated inside these core-shell particles. Since bio-nanoparticles, such as viruses and virus-like particles, heat shock protein cages, and enzyme complexes are highly organized scaffolds with robust chemical and biological properties and fascinating structural symmetries, this method allows the synthesis of hierarchically assembled particles for the purpose of drug delivery to selectively target tumor cells.



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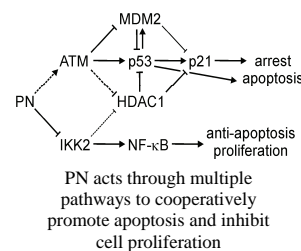
P53-16: PARTHENOLIDE, A POLYMACHIC ANTICANCER AGENT, DEPLETES HDAC1 AND MDM2 AND ACTIVATES p53 AND p21 IN BREAST CANCER CELLS

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The sesquiterpene lactone parthenolide (PN) is the primary biologically active agent in feverfew, an herbal remedy used for treating arthritis, fevers, and headaches. Because of its anti-inflammatory properties, PN is being actively investigated as a treatment for several cancers including breast cancer. In addition, PN is one of the few agents that can specifically target cancer stem cells, self-renewing multipotent progenitor cells that are naturally resistant to most conventional chemotherapeutics. To better understand its mechanism of action, we used biochemical and immunological methods to identify critical cellular proteins affected by PN treatment of breast cancer cells. We discovered

that PN promoted the rapid and extensive cellular depletion of histone deacetylase 1 (HDAC1), an important oncoprotein that epigenetically inhibits expression of p21^{WAF1/CIP1} and other cell cycle regulatory proteins. HDAC1 depletion occurred through ubiquitination and proteasomal degradation and was specific for only HDAC1 and no other histone deacetylases. PN was also found to promote the ubiquitination and inactivation of the oncogenic ubiquitin E3 ligase MDM2, which is the primary inhibitor of p53, a master regulator of many proapoptotic and cell cycle regulatory genes. Curiously, the effects on HDAC1 and MDM2 did not involve PN's known targets or signal transduction pathways. Rather, PN initiated an atypical activation of the DNA damage transducer ATM, which is absolutely required for PN's cytotoxicity. PN's cooperative effects on multiple oncoproteins and tumor suppressors could be responsible for its unique effectiveness against cancer stem cells, thus illustrating the validity of a polymachic approach for anticancer therapeutics.



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P53-17: DELIVER MULTIPLE THERAPEUTIC MOLECULES TO BREAST CANCER CELLS USING RNA NANOTECHNOLOGY AND PHI29 MOTOR COMPONENTS

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We demonstrated recently that pRNA, a component of a switchable imitating DNA-packaging motor, can be used as a building block for bottom-up assembly in nanotechnology. The pRNA's structural versatility, coupled with its ability to form dimers, trimers, hexamers and patterned superstructures via the interaction of two interlocking loops, makes it a promising tool for nanomachine fabrication, pathogen detection and gene delivery.

The polyvalent chimeric RNA complex can deliver multiple therapeutics such as siRNA to specific cells, as demonstrated in breast cancer cell lines. Incubation of the chimeric pRNA/siRNA or pRNA/ribozyme nanoparticles with breast cancer cells resulted in subsequently modulating programmed cell death. To evaluate the effectiveness of therapeutic RNA molecules in treating breast cancer, survivin was chosen as a target. Specificity was achieved via incorporating folate molecule into one subunit of the pRNA subunit. Folate is a ligand for specific targeting via receptor-mediated endocytosis. The function and specificity of pRNA/siRNA (survivin) were examined in human breast cancer cell line MCF-7, which highly expresses folate receptors on its surface. The apoptosis of breast cancer cells transfected with pRNA/siRNA (survivin) was assessed by flow cytometry analysis. Cells transfected with pRNA/siRNA (survivin) showed a much higher percentage of apoptotic cells, compared with those treated with mutant chimeric pRNA/siRNA or 5S RNA as negative controls. The effects of chimeric pRNA/siRNA on survival cells were also evaluated by cell morphology studies on another breast cancer cell line, MDA-231. After transfection, the majority of cells shrank and were detached from the cell culture plate, whereas the negative control pRNA/siRNA (mutant) did not induce cell death.

RNA three-D design, circular permutation, folding energy alteration, and nucleotide modification were applied to generate stable RNA nanoparticles with low toxicity, and to make the chimeric RNA complexes processed into siRNA by Dicer after delivery. Using such protein-free nanoparticles, which are by composition poor immunogens, as therapeutic reagents would allow for long-term administration to avoid the immune response due to repeated treatment for chronic diseases.

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P53-18: EARLY IN VITRO PASSAGES OF BREAST CANCER CELLS DIFFERENTIALLY EXPRESS THE NOVEL RETINOIC ACID RECEPTOR β-5 ISOFORM AND ARE DIFFERENTIALLY SUSCEPTIBLE TO RETINOIDS

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Background: Most studies on retinoid receptors have been focused on RARβ2 because it is expressed in normal cells but is lacking in most breast and other types of cancer. We have recently identified a novel RARβ isoform, RARβ5, which lacks A, B, and part of C-domains of RARβ2 and has a distinct promoter, P3 that does not have the retinoic acid response elements (RARE) of the RARβ2 P2 promoter (Peng et al. 2004). We hypothesized that RARβ5, contrary to RARβ2 may serve as a potential biomarker of resistance of breast cancer cells to retinoids. To test this hypothesis, we employed

for the first time early in vitro passages (#3 -12) of breast cancer (EPBC), which have been considered in biology closer to the primary tumors than the established breast cancer cell lines.

Methods: Cells from nine EPBC (selected from 29) were treated for 4 days with 1.0 mM of atRA, 9cRA, or 4-HPR and their viability/growth was determined by MTT assay. RAR β 5 and RAR β 2 were determined at mRNA level by RT-PCR and at protein level by western blotting and ICH. RAR α , RAR γ , RXR α , and RXR β were also examined by western blotting. siRNA was employed to knock down RAR β 5 transcription and to determine whether this might affect the sensitivity of tumor cells to retinoids. The response of an EPBC, which expresses RAR β 5 at mRNA and protein levels to atRA was also examined in nude mice tumor xenografts.

Results: Among nine EPBC examined, five were susceptible to atRA, four to 4-HPR, and two to 9cRA, indicating that primary breast cancer cells are differentially susceptible to retinoids. Both RAR β 5 and RAR β 2 mRNAs were differentially expressed in all EPBC, whereas at protein level, RAR β 5 was detected in 1 of 9 EPBC (BCA-2) only, which also was resistant to retinoids, both in vitro and in xenograft tumor assay. RAR β 5 suppression by siRNA in MDA-MB-231 cells increased their susceptibility to atRA, suggesting that at least in subset of breast carcinomas RAR β 5 may reflect the resistance of tumor cell to retinoids.

Conclusions: These results demonstrate that only about 50% of EPBC are susceptible to atRA and 4-HPR and 20% to 9cRA, suggesting that only subset of breast carcinomas may respond to selective retinoids. All EPBC express RAR β 5 at mRNA level, but only 1 of 9 at protein level and this EPBC was resistant to all three retinoids. These data may have clinical implication in selecting patients with breast cancer that would mostly benefit from clinical trials with retinoids.

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P53-19: SITE-DIRECTED THERAPY WITH NON-ANTICOAGULANT HEPARINS AND CHEMOTHERAPY IN BREAST CANCER

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There is substantial literature support for the use of low molecular weight heparins (LMWH) for treating coagulation disorders in cancer patients. Recent prospective and retrospective clinical trials have also demonstrated that they provide significant advantages in terms of progression-free and overall survival in certain cancers and in certain subgroups of patients. LMWH treatments are often associated with increased bleeding, constituting a dose-limiting effect. We have developed novel non-anticoagulant heparin (NACH) compounds that have minimal effects on hemostasis (El-Naggar and Mousa, U.S. Patents 6,908,907 B2, (2005), and 10, 667,216, (2003). We have evaluated them for efficacy versus tumor growth and metastasis and have begun to investigate the mechanisms involved in anti-tumor activities. Modified sulfated LMWH with weak or no anticoagulant activities were still highly effective in inhibiting angiogenesis and metastasis, demonstrating that anticoagulation is not essential for attenuation of angiogenesis or metastasis. The modified heparins were characterized with respect to their ability to release endothelial tissue factor pathway inhibitor (TFPI) and inhibit selectin-mediated interactions, molecular components that have been shown to modulate tumor growth, tumor angiogenesis, and metastasis. One of these modified heparin compounds that showed significant activity in a selectin-mediated tumor cell adhesion assay was also highly effective in reducing tumor burden in mice with MC-38 colon carcinoma and B16-BL6 melanoma (>70%) and in reducing the number of metastatic foci (>65%) in these animals. We also investigated the efficacy of NACH compounds on growth factor-induced angiogenesis in a mouse Matrigel model in which new vessel growth was quantified by measuring hemoglobin concentration extracted from the Matrigel plug. Values are Means \pm SEM. Matrigel alone: 0.57 \pm 0.12; bFGF + Matrigel: 7.27 \pm 1.18; NAC-S-S: 0.86 \pm 0.10. This sulfated compound that demonstrated no anticoagulant activity in aPTT and TEG assays reduced capillary formation to baseline levels. These data demonstrate that non-anticoagulant heparin compounds exhibit a profile of anti-tumor activities without disrupting normal hemostasis. Site-directed therapy with NACH and chemotherapy would allow for optimization of treatments in the tumor microenvironment. In studies that are currently under way, we are targeting the sites of tumor neovascularization using a biodegradable nanoparticulate system made up of a blend of MPEG-PLGA (methoxy-polyethylene glycol-poly (lactide-co-glycolide) and maleimide-PEG-PLGA. These nanoparticles have their surfaces conjugated to α v β 3 antibody and contain chemotherapeutic agent Doxorubicin, with or without NACH. Preliminary data indicate that repeated administration of sulfated non-anticoagulant heparin compound at 10 mg/kg S.C. daily for up to 14 days in conjunction with doxorubicin caused no bruising at the injection site, whereas Enoxaparin showed injection site bruising in >50% of the mice. The use of NACH agents that are co-encapsulated with chemotherapeutic agents could minimize the toxic side effects of the chemotherapy while delivering a combination of effective therapeutic agents directly to the tumor.

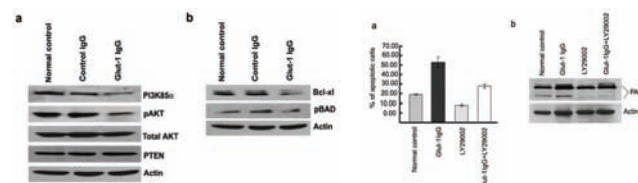
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0344.

P53-20: ANTI-GLUT-1 ANTIBODY AS A THERAPEUTIC MODALITY AGAINST BREAST CANCER

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We have previously shown that treatment with anti-Glut-1 antibody induces apoptosis and reduces apoptosis in breast cancer cell lines {Rastogi et al., 2007}

We show here that anti Glut-1 antibody induces apoptosis by an Akt-dependent mechanism. Treatment with anti-Glut-1 monoclonal antibody reduces the expression of anti-apoptotic proteins, PI3K (phosphatidylinositol-3 kinase) and Akt, essentially down-regulating this anti-apoptotic pathway commonly up-regulated in tumors. Additionally we also show that several of the anti-apoptotic proteins in the Bcl-2 family are also downregulated i.e., Bcl-xl and pBAD. Additionally, treatment with a PI3K inhibitor LY29002 decreases the apoptosis induced by the anti-glut-1 antibody as demonstrated by the PARP cleavage. These results are shown in Figure 1.



In conclusion, we show here that treatment of breast cancer cell lines with anti-Glut-1 antibody increases apoptosis by down-regulating the anti-apoptotic PI3K-Akt pathway. Experimentation is currently under way in female nude mice with implanted breast cancer cell lines to further corroborate the cell line experimentation outlined in this abstract. Updated data will be presented at the meeting.

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P53-21: ENGINEERING ANTI-EGFR ANTIBODIES FOR TREATMENT OF BREAST CANCERS WITH POOR PROGNOSIS

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Background: Monoclonal antibodies have been shown to significantly improve the response rate and prognosis of a number of cancer types, especially when combined with chemotherapy. However, basal-type breast cancers have an especially poor prognosis and no existing antibody therapy has been shown effective for this tumor subtype. Since the EGF receptor (EGFR) has been shown to be overexpressed in most of the basal-type breast cancer by immunohistochemistry, generation of highly potent anti-EGFR antibody may lead to a targeted therapy for this subtype of breast cancers. Our laboratory has successfully applied yeast display to increase the affinity of an EGFR antibody for EGFR. The engineered anti-EGFR single chain Fv antibodies exhibit enhanced anti-proliferative effect in vitro for EGFR positive breast cancer cells. The study of engagement of antibody Fc and Fc receptors indicates that engineering Fc may enhance the anti-cancer efficacy of antibodies. Mutagenesis of the interface between human Fc and human Fc receptors based on crystal structure has demonstrated that engineering the Fc can alter the potency of the immune response in vitro. To permit determination of the importance of Fc engineering in enhancing antibody efficacy in vivo, we propose to engineer the murine IgG1 Fc for enhanced binding to activating Fc receptors and reduced binding to inhibitory Fc receptors. The EGFR antibodies with wild-type and engineered Fc will be evaluated in murine xenograft models of breast cancer. Such studies may to the identification of more potent EGFR antibodies that could be used for the treatment of basal subtype breast cancers.

Hypothesis/Objectives: (1) Directed evolution by yeast display can be used to engineer the IgG Fc region for optimal binding to Fc γ activating receptors and reduced binding to inhibitory Fc receptors. (2) Anti-EGFR IgG with engineered Fc region that selectively bind Fc activating receptors will have enhanced anti-tumor activity in basal-type breast cancer xenograft models.

Specific Aims: (1) Utilize directed evolution and yeast display to engineer the murine Fc region of IgG for increased binding to activating murine Fc receptors and reduced binding to inhibitory Fc receptors. (2) Determine the benefit of using anti-EGFR IgG with enhanced differential Fc binding for treating basal type breast tumors in a murine xenograft model.

Results: Murine IgG1 Fc was cloned into a yeast display vector and successful display and functional binding to protein A demonstrated. Activating murine Fc gamma receptor III and inhibitory murine gamma receptor IIB were expressed from 293T cells and purified. Both receptors were shown to bind to yeast displayed murine Fc. The plan going forward is to measure the affinity of yeast displayed Fc for the two Fc receptors. A library of random Fc mutants will be created using error prone PCR and displayed on the yeast surface. Flow cytometry will be used to select yeast displayed

Fc that bind with greater differential binding to activating Fc receptor compared to inhibitory Fc receptor. EGFR IgG containing the engineered Fc will be cloned and expressed from CHO cells and their ability to activate ADCC in vitro measured. Mutant Fc with greater ADCC will be evaluated in vivo in a murine model of basal subtype breast cancer to determine the relationship between enhanced ADCC and anti-tumor efficacy.

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P53-22: *Clostridium perfringens* ENTEROTOXIN AS A NOVEL TARGETED THERAPEUTIC FOR BRAIN METASTASIS

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Brain metastasis is the most commonly occurring intracranial tumor whose incidence appears to be increasing. With standard therapy, the average survival time of patients is approximately 8 months, and treatment often leads to neurological dysfunction in long-term survivors, emphasizing the need for novel therapeutics. *Clostridium perfringens* enterotoxin (CPE) has recently been shown to rapidly and specifically destroy cancer cells expressing CPE receptors, claudin (CLDN)-3 and -4. Unfortunately, the utility of CPE is precluded by systemic toxicity since its receptors are expressed in numerous organs. Here, we provide the first preclinical evidence that CPE may be uniquely suited to the local treatment of brain metastasis. By immunohistochemical (IHC) analysis, CLDN-3 and -4 were expressed frequently in metastases from breast (15/18), lung (15/20), and colon (12/14) carcinoma, and infrequently in metastases from renal cell carcinoma (2/16) and melanoma (2/16). In contrast, CLDN-3 and -4 expression was absent in adjacent normal brain tissue. Further examination of the central nervous system (CNS) revealed low or undetectable levels of CLDN-3 and -4 in all regions tested by western and IHC analysis. Treatment of breast cancer cell lines (MDA-MB-468, NT2.5, MCF-7) and normal human astrocytes with CPE in vitro resulted in rapid and dose-dependent cytolysis exclusively in breast cancer cells, correlating with CLDN-3 and -4 expression. Moreover, intracranial CPE treatment of breast tumor xenografts in the brain of immunodeficient mice significantly inhibited tumor growth and increased survival, without any apparent local or systemic toxicity. These data suggest that CPE therapy may have efficacy against a wide variety of brain metastases without CNS toxicity.

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P53-23: PROGRAMMABLE MOLECULAR SENSORS FOR THE DESIGN OF INTELLIGENT THERAPEUTICS TARGETED TO BREAST CANCER CELLS

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Background, Objectives, and Methodologies: The objective of this research is to develop universal platforms for the design of integrated RNA-based regulators that modify expression levels of target genes through RNA interference pathways in response to changes in effector levels. Such platforms will have significant applications in increasing the efficacy of cancer therapeutics and form the basis of technologies that will enable targeted molecular therapies. The application of these regulator molecules as intelligent therapeutics that detect breast cancer states and trigger apoptosis in cancer cells will be demonstrated in cell culture models for breast cancer. The specific aims are to: (1) develop ligand-controlled RNA platforms for the rational design of molecular switches that regulate the expression levels of targeted genes in response to intracellular effector levels; (2) construct RNA switches that regulate cell fate decisions such as apoptosis in response to effector levels; (3) construct RNA sensors that regulate the expression of fluorescence reporter genes in response to cancer biomarkers; and (4) develop RNA switches that function as intelligent cancer therapeutic molecules that trigger apoptosis in targeted breast cancer cells.

The research methodology will follow that outlined in the specific aims. Modular platforms for the construction of integrated, ligand-regulated RNAi substrates will be developed and validated in appropriate cell lines. The response properties of these noncoding RNA molecules will be determined initially on fluorescent reporter gene targets by expressing these molecules from appropriate expression vectors. RNA regulatory sequences will be examined for desired gene silencing effects such as those triggering apoptosis. RNA sensors, or aptamers, will be generated to appropriate cancer biomarkers or effector molecules and subsequently placed into the modular regulatory platform. Standard fluorescence or apoptosis assays will be used to examine the effects of these synthetic regulatory molecules.

Results and Conclusions: We have developed a tunable and modular platform for the construction of RNA-based regulators that control the expression levels of target genes through the RNA interference pathway in response to changes in intracellular effector levels. The ability to construct integrated, small molecule-responsive, RNA-based regulatory molecules through this platform has been demonstrated. In addition, modeling tools have been developed and validated as tools for the forward design of such regulatory molecules with response properties optimized for different application-specific performance requirements. We have also established model breast cancer cell lines and vector systems for quantifiable characterization of the regulatory response of these synthetic ligand-controlled RNAi substrates. These regulatory molecules have been shown to be active in breast cancer model cell lines, as well as other cell lines.

The developed platforms have important implications for the construction of therapeutic molecules that target specific cancer cells, resulting in increased efficacy of the therapy. The properties of modularity and programmability are critical in the broad application of these molecules to different diseases with varied molecular biomarkers. This will be demonstrated in the subsequent work under this award as aptamers to breast cancer biomarkers are generated and implemented in these platforms.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0250.

DRUG RESISTANCE II

Poster Session P54

P54-1: SUSTAINED ACTIVATION OF EGFR IN BREAST CANCER CELLS RESULTS IN HIGHER KINASE ACTIVITY, LOSS OF MAP-2 AND SUBSEQUENT RESISTANCE TO DOCETAXEL

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One mechanism that contributes to the generation and maintenance of cancerous cells is the disruption of the cellular cytoskeleton. Microtubule associated proteins (MAPs) are major components of cytoskeleton proteins associated with microtubule assembly and its stabilization. MAP-2, a component of the MAP family, is a marker for neurons, and its immunoreactivity has been demonstrated in several neoplasms. We hypothesized that MAP-2 expression is deregulated in EGFR overexpressing breast cancers thus rendering them resistant to conventional therapy. We first evaluated the expression of MAP-2 in a range of breast cancer cells and found that all cell lines expressed varying degrees of MAP-2. However, when compared on the basis of their EGFR status, we observed higher expression of MAP-2 in EGFR over-expressing cells than in non-EGFR overexpressing cells both at protein and mRNA levels. To investigate whether the expression of MAP-2 is regulated by the expression of EGFR we selected two cell lines based on their responsiveness to EGF. MCF-7 and MCF-10A cells were challenged with increasing doses of EGF (25–150 ng/mL) and examined for the expression of phosphorylated EGFR. We observed that MCF-7 cells were weakly and MCF-10A cells were strongly stimulated when challenged by increasing doses of EGF. We next examined the expression of MAP-2 in cell lines challenged with EGF and observed that although MAP-2 expression increased with increasing doses of EGF, its expression was almost completely lost at concentrations >100 ng/ml of EGF treatment. This observation suggested a possible mechanism of resistance in breast cancer patients with EGFR overexpression. Based on these observations, we conclude that with constant EGF stimulation, cells lose their MAP-2 expression that is required for microtubule stabilization and subsequent cell cycle arrest. In the next series of experiments we sought to understand how MAP-2 expression would change in EGFR-expressing cells that are challenged with EGF and then treated with microtubule-disrupting agents. We found increasing resistance to growth inhibition by docetaxel in cells that were challenged with higher concentrations of EGFR (>50 ng/ml). To understand the underlying mechanism of loss of MAP-2 during sustained activation of the EGFR signaling, we hypothesized this increased loss may be occurring due to increased activity of kinases downstream of the EGFR. We utilized a kinase array to screen the status of whole-cell kinases in control and EGF-treated (100 ng/ml) cells. Interestingly, we observed increased kinase levels of several substrates that are activated by EGFR signaling. In particular, increased levels of protein kinases, mitogen-activated protein kinases (MAPK) such as JNK and Erk, were observed. These observations suggest under conditions of increased kinase activity, MAP-2 is hyperphosphorylated and eventually degraded resulting in loss of MAP-2 from the system. Thus, loss of MAP-2 expression in breast cancer cells during sustained activation of the EGFR results in resistance to docetaxel. This suggested that overexpression of EGFR signaling in breast cancers could, in fact, be responsible for resistance to therapeutic agents. The loss of MAP-2 expression could have implications in treatment of breast cancers overexpressing the EGFR and exhibiting resistance to conventional therapy.

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P54-2: 3,3'-DIINDOLYLMETHANE (DIM) ABROGATES THE CHEMORESISTANCE OF TAXOTERE IN ErbB2 OVEREXPRESSION BREAST CANCER BY DOWNREGULATION OF ErbB SIGNALING

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About 25% of all breast cancers overexpress the HER-2/erbB2 oncogene; these tumors are aggressive and tend to be resistant to standard chemotherapeutic drugs. Overexpression of HER-2/erbB2 is thought to be a mechanism for the resistance to chemotherapy. DIM is a naturally occurring active component of cruciferous vegetables. Previously, we showed that DIM enhanced the cytotoxic effects of taxol in breast cancer cells. In this study, we investigated the mechanisms by which DIM overcomes the resistance of HER-2 overexpressing breast cancers to taxanes, by examining the effectiveness of DIM in combination with docetaxel (Taxotere) on the activation of the erbB family of receptors and their downstream signaling pathways. DIM inhibited the docetaxel-induced protein expression and activation of epidermal growth factor receptor (EGFR)/erbB1/HER-1, erbB-2/neu/HER-2, erbB3/HER-3, and ERK1/2 in 435eB and BT474 cells as determined by western blot. Pretreatment of 435eB cells with DIM for 3 hr produced a greater growth inhibitory and apoptotic effect than either treatment alone or the higher concentration of docetaxel. DIM-induced apoptosis in 435eB cells by increasing phosphorylation of p38. To study the effects of DIM and docetaxel on tumor growth, 435eB cells (1×10^6) were injected into the mammary fat pad of 6- to 8-week old nude mice. When mammary tumors were between 100 to 150 mm³, mice (n=5) were started on the AIN-93M control diet or AIN-93M diet supplemented with 150 ppm absorption-enhanced DIM (BioResponse, Boulder, Colorado), AIN-93 diet plus docetaxel treatment, or AIN-93 diet supplemented with DIM plus docetaxel treatment. We observed that DIM in combination with docetaxel decreased tumor growth

rate to 1% a day compared to tumors from control mice that grew 10% a day. The findings of this study demonstrate that DIM enhanced the response of HER-2 overexpressing breast cancer to docetaxel therapy both in vitro and in vivo. The results of this study provide an innovative approach to increase responsiveness of HER-2 overexpressing breast cancer to the taxanes by using the dietary component DIM that may result in patients receiving lower doses of the drug and less harmful side effects.

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P54-3: SPHINGOLIPIDS UPREGULATE EXPRESSION OF THE MULTIDRUG RESISTANCE GENE MDRI IN BREAST CANCER CELLS

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One of the most persistent factors accounting for the continuing mortality in cancer patients is the development of multidrug resistance (MDR). We have previously shown that both glucosylceramide synthase (GCS) and P-glycoprotein (P-gp) are overexpressed in MCF-7-AdrR cells, a multidrug resistant human breast cancer cell line. MCF-7-AdrR cells transfected with GCS antisense showed enhanced ceramide formation compared with parent MCF-7-AdrR cells when challenged with paclitaxel, and a 10-fold increase in paclitaxel and vinblastine uptake. In assessing P-gp status in the GCS antisense transfectants, we observed a dramatic decrease in the level of MDRI expression, (80% down by RT-PCR), which translated into a similar decrease in P-gp protein levels (Western-blot). These findings demonstrate an interesting yet ambiguous relationship between GCS, which regulates ceramide metabolism, and the expression of P-gp. To gain insight into the relationship of GCS and MDRI, we studied the influence of sphingolipids on MDRI expression. Ceramide is converted to glucosylceramide by GCS. When wild-type breast cancer cells, MCF-7 and MDA-MB-231, were exposed to a low concentration of [¹⁴C]C6-ceramide (0.2 µg/ml medium), cells converted it to sphingomyelin via sphingomyelin synthase. However, when these cells were challenged with a higher concentration of [¹⁴C]C6-ceramide (5.0 µg/ml), it was primarily glycosylated to GC via GCS. This demonstrates that cells subjected to high ceramide levels utilize a salvage pathway to eliminate apoptosis-inducing lipid. Chronic exposure of MDA-MB-231 cells to short-chain ceramides (5.0 µg/ml medium) for extended periods induced a dramatic increase in MDRI mRNA levels, and also elicited expression of P-gp. When tested in cytotoxicity assays, these ceramide-grown cells were more resistant to adriamycin and paclitaxel. This study shows that although cells challenged with high levels of ceramide utilize a glycosylation route to limit ceramide's residence time, this action promotes enhanced expression of the multidrug resistant phenotype in cancer cells through what we propose is a GC intermediate.

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P54-4: MECHANISMS OF CELL DEATH IN ACQUIRED TAXANE RESISTANCE MODELS

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Treatment with taxanes (paclitaxel or docetaxel) is often the therapy of choice for women with breast cancer. In most cases, the taxanes can arrest cell proliferation at the G2/M phase and cause cell death. However, some tumors develop resistance during the course of treatment, which is a major concern for both patients and physicians. Several mechanisms have been proposed to explain how resistance to the taxanes occurs, but this phenomenon remains incompletely understood. We hypothesize that changes in cell death signaling, through apoptotic and nonapoptotic pathways, are responsible for taxane resistance in breast cancer. Using the MDA-MB-231 breast cancer cell line model, we selected cells that are resistant to either docetaxel (DocR) or paclitaxel (PacR). Subsequently, we have studied their baseline levels of apoptosis and their response to IC₅₀ concentrations of these drugs by several measures: SubG1 cell cycle fraction, annexin V staining, mitochondrial permeability, and caspase activation. Generally, increases in the SubG1 fraction, early and middle apoptosis, necrosis, and mitochondrial permeability are seen in sensitive MDA-MB-231 cells in response to either taxane. Interestingly, the DocR and PacR cell lines both exhibit higher baseline levels of these apoptotic measures than the sensitive cell line, but in general, IC₅₀ levels of these drugs can no longer induce additional apoptosis with the exception of the SubG1 fraction. The sensitive MDA-MB-231 and resistant DocR and PacR cells all appear to activate the caspases in their mechanisms of cell death with and without drug treatments at slightly altered levels. Given our preliminary data, we propose to further investigate apoptotic and alternative cell death evasion pathways seen in both MDA-MB-231 and MCF7 breast cancer cells and compare them to levels seen in clinical samples of human breast cancer tissue from patients who were responsive and nonresponsive to taxane treatment. Our studies aim to improve the understanding of how different mechanisms of cell death evasion contribute to the resistance phenotype. Ultimately, our studies aim not only to improve our understanding of taxane resistance on the bench but may also

provide the framework for formulation of novel therapies that may improve the success of taxane therapy in women with breast cancer in the clinic.

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P54-5: FATTY ACID SYNTHASE, A NOVEL TARGET IN THE TREATMENT OF DRUG-RESISTANT BREAST CANCERS

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Multidrug resistance (MDR) is a major problem in successful cancer chemotherapy. Various mechanisms of resistance such as ABC transporter-mediated drug efflux have been discovered using established model cancer cell lines. While characterizing a drug-resistant breast cancer cell line, MCF7/AdVp3000, we found surprisingly that fatty acid synthase (FASN) is overexpressed. In this study, we demonstrated that ectopic overexpression of FASN indeed increases drug resistance in breast cancer cell line MCF7, while reducing the FASN expression increased the drug sensitivity in breast cancer cell lines MCF7/AdVp3000 and MDA-MB-468, but not in the normal breast epithelial cell line MCF10A1. The FASN-mediated drug resistance appears to be due to the decrease in drug-induced apoptosis via Caspase-8 dependent pathway. The detailed mechanisms of FASN inhibition of drug-induced apoptosis is under investigation. Together with previous findings that FASN is a marker predicting poor prognosis for breast cancer patients, our findings suggest that FASN may be an ideal target for chemo-sensitization in breast cancer chemotherapy.

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P54-6: HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF THE P-GLYCOPROTEIN HOMOLOGUE FROM *Plasmodium falciparum*

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The multidrug resistance (MDR) phenomenon seen in cancer cells bears similarity to that found in malaria, with simultaneous resistance to multiple classes of drugs occurring within the same strain. Some MDR in cancer has been attributed to P-glycoprotein (Pgp), a member of the ATP-Binding Cassette (ABC) superfamily that has been hypothesized to act as a drug efflux pump. The *Plasmodium falciparum* multidrug resistance protein (PfMDR1) is a putative 12-transmembrane domain, 2 nucleotide binding domain protein that bears high homology to Pgp. Using codon optimization, we have designed and constructed a yeast-optimized version of the wild-type *pfmdr1* gene and have successfully overexpressed PfMDR1 protein in *P. pastoris* yeast. The protein is well expressed in either full-length form or as two separate half transporters, is well localized to the yeast plasma membrane, and is fully functional as evidenced by ATPase activity measurements. We have also expressed mutants that have previously been hypothesized to influence drug resistance in parasites. Using purified plasma membrane fractions, we have analyzed antimalarial drug effects on ATPase activity for wild-type versus mutant proteins and we have found both some interesting parallels and differences with Pgp.

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P54-7: OLIGOMERIZATION DOMAIN OF THE MULTIDRUG RESISTANCE-ASSOCIATED TRANSPORTER ABCG2 AND ITS DOMINANT INHIBITORY ACTIVITY

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Overexpression of human ATP-binding cassette transporter ABCG2 in cancer cells causes multidrug resistance by effluxing anticancer drugs. ABCG2 is considered as a half transporter and is thought to function as a homodimer. However, recent evidence suggests that it may exist as a higher form of oligomer consisting of 12 subunits. In this study, we mapped the oligomerization domain of human ABCG2 to its transmembrane domain consisting of TM5-loop-TM6. This oligomerization domain, when expressed alone in HEK293 cells, also forms a homododecamer. Furthermore, this domain has activity that inhibits drug efflux and resistance function of the full-length ABCG2 likely by disrupting the formation of the homo-oligomeric full-length ABCG2. These findings suggest that human ABCG2 may exist and work as a homo-oligomer by interactions located in TM5-loop-TM6, and that ABCG2 oligomerization may be used as a target for therapeutic development to circumvent ABCG2-mediated drug resistance in cancer treatment.

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P54-8: ELUCIDATING MECHANISMS OF FARNESYLTRANSFERASE INHIBITOR ACTION AND RESISTANCE IN BREAST CANCER BY BIOLUMINESCENCE IMAGING

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Farnesyltransferase inhibitors (FTIs) block the post-translational processing of signaling proteins, such as Ras, that have key roles in breast cancer biology. In phase II trials, FTIs have exhibited clinical benefit toward a subset of breast cancer patients. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. To overcome these hurdles, mechanisms determining whether breast cancer tumors are FTI-sensitive or -resistant *in vivo* must be understood. These mechanisms remain elusive because signaling networks regulating proliferation, survival, and migration of breast cancer cells *in vitro* versus *in vivo* can be strikingly different, and because conventional biochemical means of detecting FTI action in tumors are insensitive, invasive, or correlate poorly with FTI action. Therefore, novel means of elucidating mechanisms of FTI activity in tumors are required.

Accordingly, this recently funded project will develop molecular imaging strategies that for the first time specifically detect the ability of FTIs to inhibit farnesylation in tumors of living animals. This novel imaging strategy will be coupled with molecular screening methods to identify mechanisms of FTI sensitivity and resistance in tumors. Our initial proposed imaging strategy uses a chimeric transcription factor fused to H-Ras that localizes to the nucleus upon inhibition of farnesylation. The deprenylation-activated chimeric transcription factor consists of a Gal4 DNA binding domain, a VP16 transcriptional activation domain, a nuclear localization sequence (NLS), and either the wild-type or prenylation site mutant form of H-Ras. When unprenylated, the H-Ras fusion chimeras will bind a Gal4 promoter driving expression of firefly luciferase, a reporter that can be readily imaged in cells and animals with an ultrasensitive, cooled CCD camera. Thus, luciferase will be expressed when farnesylation of H-Ras, a key signaling protein, is blocked. The luciferase reporter system will be incorporated into human breast cancer cell lines and validated extensively *in vitro* and in breast cancer xenograft models. The system will be used to compare FTI action toward tumor xenografts derived from breast cancer cell lines that are sensitive versus resistant to FTIs *in vitro*.

This strategy should offer the opportunity to visualize over time the action of FTIs toward specific, biologically relevant isoprenoid-dependent proteins in tumors of living animals. We hope to address whether FTIs exert their adjuvant chemotherapeutic action by inhibiting farnesylation of specific, biologically relevant targets such as H-Ras, and over the long term, to define the mechanisms responsible for FTI sensitivity and insensitivity *in vivo*.

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P54-9: INCREASED EXPRESSION OF c-Met IN FULVESTRANT-RESISTANT BREAST CANCER CELLS

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c-Met is the tyrosine kinase receptor for scatter factor/hepatocyte growth factor. Expression of c-Met is associated with poorer outcome in breast cancer. Activation of c-Met signaling can lead to cell scattering, angiogenesis, proliferation, enhanced cell motility, invasion, and eventual metastasis. Fulvestrant (Faslodex[®], ICI 182,780) is a pure antiestrogen approved by FDA as a second-line endocrine therapy for the treatment of ER α -positive breast cancer. Inevitably, breast cancer tumors will overcome the effect of fulvestrant and will progress. To identify potential new therapeutic targets for fulvestrant-resistant breast cancer, we have developed a fulvestrant-resistant cell model (MCF-7/F) derived from MCF-7 ER α -positive human breast cancer cells. MCF-7/F cells become ER α negative and hormone independent. Interestingly, MCF-7/F cells are highly migratory and invasive in *in vitro* assays. Concomitantly, MCF-7/F cells differentially highly express c-Met at both mRNA and proteins levels and have significantly higher c-Met promoter activity as compared to the MCF-7 control cell. The level of c-Met protein and c-Met promoter activity is comparable to the ER α -negative and metastatic breast cancer cell line MDA-MB-231 cells. These results suggest that c-Met might play a critical role in the more aggressive phenotype of fulvestrant-resistant MCF-7/F cells. Moreover, c-Met could serve as a potential target for manipulation of fulvestrant-resistant MCF-7 cells.

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P54-10: TARGETING AUTOPHAGIC SURVIVAL PATHWAY SENSITIZES HUMAN BREAST CANCER CELLS TO GROWTH FACTOR ANTAGONISTS

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Malignant cancer cells often face metabolic stresses such as nutrient, growth factor or oxygen scarcity. We previously reported that cancer cells utilized autophagy, a highly regulated cellular degradation process, as a survival strategy to cope with metabolic stress. Elongation factor-2 kinase (eEF-2 kinase), a protein synthesis regulator, phosphorylates and inactivates eEF-2, thereby terminating peptide chain elongation and inhibiting protein synthesis. Since protein synthesis is a major energy-consuming process, down-regulation of this process by activating eEF-2 kinase may be used by cancer cells as an energy-saving survival mechanism. In the current study, we tested the hypothesis that eEF-2 kinase plays a critical role in the ability of breast cancer cells to survive growth factor/nutrient deprivation. Autophagy activity was determined by measuring the formation of LC3 II, and eEF-2 kinase activity by measuring phosphor-eEF-2 using immunoblot analyses. We found that eEF-2 kinase and autophagy were activated following starvation treatment in human breast cancer cell line MCF7. Nutrient starvation also decreased mTOR activity as evidenced by the decreases of phosphor-S6 kinase and phosphor-4EBP1, and reduced the incorporation rate of 35S-methionine, indicating protein synthesis was inhibited. Silencing of eEF-2 kinase by RNA interference (RNAi) relieved the inhibition of protein synthesis and resulted in a greater reduction of cellular ATP. eEF-2 kinase-targeted RNAi also blunted autophagic response of the tumor cells. Inhibition of autophagy by knockdown of eEF-2 kinase or autophagy-related gene Beclin-1 impeded cell growth in serum/nutrient-deprived cultures and handicapped cell survival. These results indicate that in response to nutrient/growth factor deprivation breast cancer cells activates eEF-2 kinase and autophagy to decrease protein synthesis and regenerate ATP, and that inhibition of eEF-2 kinase renders cells continue to elongate peptide, deplete ATP, and impairs cancer cell survival under metabolic stress. Furthermore, we determined whether inhibition of autophagy sensitized breast cancer cells to growth factor antagonists. Synergistic effect on cell growth inhibition was observed from combination of a small molecule EGFR/ErbB-2 inhibitor with an autophagy inhibitor 3-methyladenine in SKBR3 and MDAMB468 cells (combination index values at ED50 0.6279 and 0.7879, respectively). Inhibition of autophagy by knockdown of eEF-2 kinase or Beclin-1 sensitized SKBR3 and MDAMB468 cells to the EGFR/ErbB-2 inhibitor and the mTOR inhibitor rapamycin. These results provide new evidence that activation of eEF-2 kinase and autophagy plays protective role for cancer cells under metabolic stress, and that targeting autophagic survival may represent a novel approach to sensitizing cancer cells to growth factor antagonists.

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P54-11: CHEMOMODULATION OF DOXORUBICIN RESISTANCE IN MCF-7 BREAST CANCER CELLS BY LOPERAMIDE

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Multidrug resistance of tumor cells can be a problem in breast cancer treatment with drugs such as doxorubicin. There is a need for safe nontoxic agents for reversing multidrug resistance of tumors. The anti-diarrhea medication loperamide (4-[p-chlorophenyl]-4-hydroxy-N,N-dimethyl- α -diphenyl-piperidinebutyramide) was tested as a chemosensitizer of multidrug resistant MCF-7 clone 10.3 human breast cancer cells toward doxorubicin, using MTT assay for cell viability. MCF-7 clone 10.3 cells were nearly 200-fold more resistant to doxorubicin than parental wild-type MCF-7 cells on the basis of MTT assay. The IC₅₀ of doxorubicin decreased from 50 μ M to 3 μ M and 1 μ M, respectively, in the presence of 10 μ M and 20 μ M loperamide, after 72 hr of continuous exposure of MCF-7 clone 10.3 cells to drug(s). Loperamide inhibited cell proliferation even in the absence of doxorubicin. Treatment of MCF-7 clone 10.3 cells with 10 μ M and 20 μ M loperamide for 72 hr caused 20% and 40% decrease in cell proliferation relative to untreated control. Flow cytometry of cells treated with doxorubicin (10 μ M and 20 μ M) with and without loperamide (10 μ M and 20 μ M), showed increased accumulation of doxorubicin by MCF-7 clone 10.3 cells in the presence of loperamide. Accumulation of the relatively nontoxic fluorescent dye Rhodamine 123 was also monitored after incubation of MCF-7/MDR cells for 3 hr at 37°C with 100 ng/mL or 200 ng/mL of the dye alone or in combination with loperamide at 10 μ M and 20 μ M level. Fluorescence intensity of cells was measured by using flow cytometer. Cells were treated with loperamide (10 μ M and 20 μ M) for 3, 24, and 96 hr and analyzed for the expression of mRNA related to proteins associated with drug resistance. The effect of loperamide (10 μ M and 20 μ M) on the expression of mRNA for the drug resistance related protein MRP in MCF-7 clone 10.3 cells was studied using RT-PCR. The mRNA for MRP decreased even after a 3-hr treatment with loperamide. This decrease persisted for the next 4 days. Loperamide, which is an over-the-counter anti-diarrhea agent, sensitizes MCF-7 clone 10.3 by inhibiting MRP production and increasing cellular uptake and retention of doxorubicin.

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P54-12: A SINGLE NUCLEOTIDE POLYMORPHISM IN THE MDM2 PROMOTER (SNP 309) ALTERS THE SENSITIVITY TO TOPOISOMERASE II-TARGETING DRUGS

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A single nucleotide polymorphism SNP309 (T→G) in the MDM2 promoter creates a high affinity Sp1 binding site and increases the expression of MDM2 mRNA and protein. Approximately 40% of the populations harbor at least one variant allele and 12%–17% are homozygous G/G at codon 309. This MDM2 SNP increases susceptibility to cancer and decreases the response of cancer cells to certain forms of treatment such as radiation therapy and DNA-damaging drugs. Topoisomerase II-targeting agents are commonly used chemotherapeutic drugs with a broad spectrum of activity. However, resistance to topoisomerase II poisons limits their effectiveness. We demonstrate that MDM2 SNP309 rendered a panel of cancer cell lines that are homozygous for SNP309 selectively resistant (~10-fold) to certain topoisomerase II-targeting chemotherapeutic drugs (etoposide, mitoxantrone, amsacrine, and ellipticine). The mechanism underlying this observation was Mdm-2 mediated downregulation of topoisomerase II; upon drug exposure, MDM2 bound to topoisomerase II and resulted in decreased cellular enzyme content. Knockdown of MDM2 by RNA interference stabilized topoisomerase IIa and decreased resistance to topoisomerase II-targeting drugs. Thus, MDM2 SNP309 (T→G) may represent a relatively common, previously unappreciated determinant of drug sensitivity. Given the frequency of SNP 309 in the general population (40% in heterozygous T/G and 12% in homozygous G/G condition), our observation may have important implications for the individualization of cancer chemotherapy. Studies of the clinical implication of this observation are in progress.

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P54-13: ANALYSIS OF IRS EXPRESSION AND FUNCTION IN HER2-POSITIVE BREAST CANCER

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Overexpression of HER2 occurs in 20%–30% of human breast cancer tumors, and it is associated with aggressive tumor behavior and poor disease-free survival. Although Herceptin® (trastuzumab) improves patient response to chemotherapy and overall survival, the fact that not all HER2-expressing breast cancer tumors respond to Herceptin treatment, and most tumors eventually develop resistance to this drug, underscores the need for additional research into how HER2 functions to promote aggressive behavior in tumors. Recent reports have implicated the IGF-1 signaling pathway in both the mechanism of HER2 action and in resistance to Herceptin. The insulin receptor substrate (IRS) proteins are the major downstream effectors of the IGF-1 receptor, and they play a critical role in determining the cellular response to IGF-1 stimulation. Although IRS-1 and IRS-2 are homologous, results from our own work have revealed distinct functions for these adaptor proteins. Specifically, IRS-1 suppresses metastasis whereas IRS-2 promotes metastasis. Our findings, obtained using IRS-deficient mice and the PyV-MT mouse model of mammary tumor progression, demonstrate that the expression of IRS-1 and IRS-2 can significantly impact the outcome of IGF-1R signaling in breast cancer. In light of the evidence for crosstalk between the IGF-1R and HER2, we hypothesize that the IRS proteins may be signaling modifiers in the 20%–30% of patients with tumors that overexpress the HER2 receptor and that they contribute to Herceptin resistance that results from compensatory signaling through the IGF-1R. If so, the IRS proteins could be predictive indicators of treatment response to Herceptin in patients who are candidates for this targeted therapy. To address the connection between IRS expression and HER2 function, we have initiated in vitro studies to assess the response of HER2-overexpressing cells to Herceptin treatment after manipulation of IRS expression. HER2-overexpressing cell lines SKBR3 and BT474 cells are being used for these studies. We have also initiated studies to assess the expression of the IRS proteins in HER2-positive human tumors. A pilot study has revealed differences in both the intracellular localization and overall expression patterns of IRS-1 and IRS-2 in normal breast tissue and tumors. In normal breast ducts and lobules, as well as in benign breast disease, both nuclear and cytoplasmic staining was observed for IRS-1. IRS-1 staining persisted in low-moderate grade HER2-negative tumors where it was localized primarily in the nucleus. A novel observation from our study is that IRS-1 staining was weak or absent in high-grade, HER2+ tumors, suggesting a negative correlation between IRS-1 and HER2 expression. IRS-2 staining was primarily membranous for both normal breast tissue and tumors. In normal breast, IRS-2 staining was strongest in the myoepithelial cell layer with weak staining observed in the luminal epithelial cells. IRS-2 staining increased in DCIS, and strong (++ - +++) IRS-2 staining was observed in most high-grade tumors, including HER2+ tumors. Taken together, our results indicate that the balance of IRS-1 to IRS-2 expression shifts to favor IRS-2 in HER2-positive tumors, which may contribute to the aggressive nature of these tumors. Overall, the long-term goal of our studies is to determine if IRS expression correlates with resistance to Herceptin therapy and with the aggressive behavior of these tumors.

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P54-14: MET RECEPTOR CONTRIBUTES TO TRASTUZUMAB RESISTANCE OF HER2 OVEREXPRESSING BREAST CANCER CELLS

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Her2 is overexpressed in 20%–30% of breast tumors and correlates with reduced disease-free and overall patient survival. Trastuzumab, a humanized monoclonal antibody against Her2, represents the first Her2 targeted therapy that significantly decreases the risk of relapse and prolongs patient survival. However, resistance to trastuzumab represents a significant barrier to the effective treatment of Her2 (+) breast cancer. The Met receptor tyrosine kinase is aberrantly expressed in breast cancer and also predicts poor patient prognosis. In this study, we find that Met is frequently expressed in Her2 overexpressing breast cancer cells as well as Her2 (+) breast cancer and that constitutive Met kinase activity contributes to Her2 phosphorylation. Met and Her2 cooperate in breast cancer cells to enhance cell proliferation through complementary downstream cell signaling. Importantly, the Met receptor also contributes to trastuzumab resistance, as inhibition of Met sensitizes cells to trastuzumab-mediated growth inhibition, while Met activation protects cells against trastuzumab by abrogating p27 induction. Remarkably, Her2 overexpressing breast cancer cells rapidly up-regulate Met expression following trastuzumab treatment, essentially promoting their own resistance. Our study suggests that a subset of Her2 (+) patients may benefit from combined inhibition of Her2 and Met.

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P54-15: CALMODULIN-MEDIATED ESTROGEN RECEPTOR α ACTIVATION AND ANTIESTROGEN RESISTANCE

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Estrogens and estrogen receptor alpha (ER α) are central to estrogen-dependent breast cell carcinoma induction and proliferation. ER α is the principal target for systemic endocrine/antiestrogen therapy, underscoring its biological relevance and medical importance. Recently, it has been established that calcium-dependent activation by calmodulin (CaM) is essential for estrogen-dependent ER α activity and that the active species is the CaM-ER α complex (Li L, Li Z, and Sacks DB. 2005. *J. Biol. Chem.* 280, 13097-104.). CaM also binds tightly to antiestrogens, including the most widely used chemotherapeutic agent for estrogen-dependent breast cancers, tamoxifen (TAM). The therapeutic effects of antiestrogens, like those of other CaM antagonists, are indicated to be due, in part, to the direct interaction with CaM. Oxidative stress—estrogen-induced oxidative stress and constitutive oxidative stress—is indicated in estrogen-dependent breast cancer tissues. Oxidative stress is also implicated as mediating development of resistance of breast cancers to antiestrogens. TAM is also implicated in inducing a potent oxidative stress response in breast cancer tissue. It has been demonstrated in other oxidatively stressed tissues (senescent brain) that increased levels of reactive oxygen species and the failure of cellular repair mechanisms conspire to cause accumulation of oxidized CaM species (where one or more of the nine methionine residues are oxidized to the sulfoxides), altering intracellular calcium homeostasis. Oxidation of CaM can reduce its ability to activate some target proteins without necessarily reducing binding affinity. Because there are nine methionine residues in CaM, most of which interact with the CaM binding domains of target proteins, the effects of oxidation can be specific for particular methionine residues. Our objectives include establishing the molecular mechanism, including the structural details, by which CaM activates estradiol-dependent ER α transcription and defining the role of oxidative stress in mediating CaM-ER α and CaM-antiestrogen interactions. Toward these goals, we have localized the CaM binding region of ER α to a 25 amino acid segment in the hinge region of ER α . We have initiated studies using NMR spectroscopy to determine the structure of the complex of CaM with this CaM binding region of ER α . To date, based on chemical shift changes, we have found the collapse of CaM around the ER α CaM

binding domain is much less dramatic than observed for complexes of CaM with prototypical binding domains and that relatively large structural changes occur in the C-terminal domain of CaM. Oxidation of the methionine residues in CaM eliminates binding to TAM and hydroxy-TAM. TAM binding to CaM with all methionine residues replaced by leucine is unaffected by the leucine substitutions. These results suggest that CaM bound to ER α is more extended structurally compared to typical CaM complexes and signify important structural changes in the C-terminal binding pocket of CaM. Oxidation of methionine residues in CaM results in polarity changes that decrease the affinity of CaM for hydrophobic drugs. The results will be important for a comprehensive understanding of the principles governing CaM activation of ER α and the link between oxidative stress and development of antiestrogen resistance in order to aid in the design and development of a new pharmaceuticals to treat breast cancers.

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P54-16: REGULATION OF DRUG RESISTANCE BY CYCLIN C

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Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxides (O₂⁻) or free hydroxyl radicals (OH[•]) are derived from both internal and external sources. ROS attack multiple cellular targets including lipids, proteins, and DNA. Due to the variety of cellular targets and the prevalence of ROS production, it is not surprising that many human diseases including cancers are linked to oxidative stress. Following transformation, neoplastic cells are confronted with a variety of stress conditions (e.g., hypoxia due to poor vascularization) as they attempt to form tumors. Several studies have found a compelling connection between the aberrant overexpression of heat shock genes (HSPs) and the aggressiveness of many cancers including breast and ovarian. However, the stress response has also been implicated in sensitizing transformed cells to undergo apoptosis when treated with chemotherapeutic drugs. Therefore, depending on the level and type of activation, the stress response can either promote the killing effects of drugs or protect against them. Understanding at the molecular level how the stress response is activated and how this stimulation remodels the gene expression program of the cell may provide important insight into how these signals are applied and may allow more productive use of current chemotherapy regimens.

This study focuses on the role of cyclin C and its kinase partner Cdk8. Studies in yeast revealed that cyclin C-Cdk8p are transcriptional repressors that regulate stress response genes. To relieve this repression, cyclin C is destroyed in cultures subjected to a variety of stressors (e.g., heat shock and oxidative stress). Given the high conservation of the cyclin C and Cdk8 protein families, and their universal association with the RNA polymerase holoenzyme, we tested whether the human cyclin C (HcycC) was also down-regulated in cell culture following heat shock or oxidative stress. These experiments revealed that, similar to yeast, HcycC levels are reduced during following H₂O₂ treatment in several cell lines including breast (MCF7) and kidney (HEK293). Subsequent studies have shown that oxidative stress-induced destruction of cyclin C requires the Slit2 MAP kinase pathway. Similarly, inactivation of the ERK pathway protected HcycC from oxidative stress-induced destruction. These findings suggest that stress-induced destruction of cyclin C is a highly conserved response.

In yeast, cyclin C regulation is important for the cellular response to stress. For example, mutants that fail to destroy cyclin C are hypersensitive to ROS-induced apoptosis. Conversely, deleting cyclin C protects the cell from oxidative stress. Interestingly, the human cyclin C locus has been shown to be deleted in many types of cancer including breast and ovarian. These findings, when combined with the yeast results, suggest a model in which loss of cyclin C activity may protect tumor cells from many treatment regimens that employ ROS as a killing agent (e.g., bleomycin and radiation). To test this model, knockdown and knockout experiments are currently under way in human and mouse models to determine the role of cyclin C in regulating the stress response and drug sensitivity in mammals.

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BIOMARKERS II

Poster Session P55

P55-1: ALTERATION OF TOPOISOMERASE II- α GENE IN HUMAN BREAST CANCER AND ITS ASSOCIATION WITH RESPONSIVENESS TO ANTHRACYCLINE-CONTAINING CHEMOTHERAPY

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Background: Approximately 35% of human breast cancers with HER2 gene amplification are also co-amplified for the topoisomerase II- α (TOP2 α) gene that encodes an enzyme that is a known target for anthracyclines. Current data indicate that TOP2 α gene amplification, not HER2 amplification, is the predictive marker for responsiveness to anthracyclines in breast cancer.

Methods: Using fluorescence in situ hybridization (FISH), we evaluated a "test set" of tumor tissues from patients with metastatic breast cancer who were treated on a clinical trial (H0648g) of chemotherapy with or without trastuzumab (Herceptin[®]) for amplification of both the TOP2 α and HER2 genes. We then analyzed the respective correlation of these two alterations with clinical outcomes. To validate observations in this "test set," we analyzed specimens from two subsequent, large, randomized adjuvant clinical trials of anthracycline-containing chemotherapy, one in patients with HER 2 amplification (BCIRG 006) treated with trastuzumab-based regimens and one in patients without HER 2 amplification (BCIRG 005) treated with chemotherapy alone. This larger "validation set" was used to again compare HER2 and TOP2 α gene amplification status with response to various therapies.

Results: The "test set" demonstrated that cases containing HER2/TOP2 α gene co-amplification treated with doxorubicin, cyclophosphamide (AC), and trastuzumab (ACT) had a longer progression-free survival than those treated with AC alone ($p = 0.03$). Of note, patients treated with AC alone whose tumors also had HER2/TOP2 α co-amplification had a similar statistically significant improvement in survival compared to those with only HER2 amplification ($p = 0.004$). Conversely, in patients treated with paclitaxel chemotherapy, HER2/TOP2 α co-amplification was not associated with improved clinical outcomes. Similarly, cases from the HER2-amplified BCIRG 006 "validation set," containing HER2/TOP2 α co-amplification had significantly improved survival if treated with anthracycline-containing chemotherapy alone when compared to cancers lacking TOP2 α amplification. All cases from the HER2 nonamplified (BCIRG 005) component of the "validation set" failed to show any evidence of TOP2 α amplification while 3% had evidence of TOP2 α deletion.

Conclusions: Although the H0648g "test set" analysis was limited by the number of available specimens and was considered hypothesis generating, findings from the larger BCIRG005/006 "validation set" confirm that it is TOP2 α gene co-amplification and not HER2 amplification alone that is the clinically useful predictive marker of responsiveness to anthracycline-based chemotherapy in human breast cancers.

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P55-2: GALECTIN-3 CLEAVAGE BY MATRIX METALLOPROTEINASES IN BREAST TUMOR PROGRESSION AND ITS USE AS A SURROGATE DIAGNOSTIC MARKER OF MMPs ACTIVITY

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Galectin-3 has previously been reported to be involved in various biological phenomena including cell growth, adhesion, angiogenesis, and apoptosis. It is a widely expressed protein in various tumor cells, and its expression is correlated with tumorigenesis, tumor progression, and metastasis. Galectin-3 has a unique chimera type structure consisting of a carbohydrate-binding domain characteristic of the galectin family and a collagen a-like domain susceptible to cleavage by matrix metalloproteinases at Ala⁶²-Tyr⁶³. A single nuclear polymorphism (SNP) in galectin-3 gene substituting Histidine 64 with Proline was identified. To analyze the functional significance of this SNP (rs4644), we substituted the amino acid H⁶⁴ with P and expressed them in recombinant form. The P⁶⁴ containing protein showed resistance to cleavage by MMPs. Both galectin-3 variants were expressed in galectin-3 null breast cancer cell line BT-549 by stable transfections. The cells expressing P⁶⁴ variant showed an altered morphology, reduced chemotaxis and chemo-invasion, abrogated resistance to apoptosis, and reduced tumor-forming potential and angiogenesis (the functions regulated by extracellular galectin-3) as compared to the H⁶⁴ galectin-3 harboring cells. On the other hand, the functions regulated by intracellular galectin-3, like cell proliferation and an-

chorage-independent growth efficiency remained unchanged suggesting that the extracellular cleavage of secreted galectin-3 H⁶⁴ by MMPs may play a significant role during tumor development/progression. To analyze if this cleavage could also be observed in vivo, tissue array consisting of the progressive stages of breast cancer was stained with galectin-3 monoclonal and polyclonal antibodies to selectively differentiate between the intact and cleaved galectin-3. Our results showed that in the normal breast ducts no cleavage could be observed. In lobular hyperplasia, the intact protein could be detected on the lobular side of the ducts whereas the cleaved protein could be detected adjacent to the stroma. In ductal carcinoma in situ (DCIS), the full-length protein could be detected in a few epithelial cells while the cleaved protein could be seen in the stroma. In the infiltrating carcinomas, the invasive cell clusters and the surrounding stroma were positive for cleaved protein indicating that cleavage of galectin-3 is vital for the progression of breast cancer. Next, we utilized a mouse model of human DCIS of the breast to analyze whether these results could be translated as a diagnostic tool to identify the activity of MMPs in vivo. Part of the xenograft was fresh frozen to perform in situ zymography while the other part was paraffin-embedded and stained with galectin-3 monoclonal and polyclonal antibodies. The results show an overlap between the MMP activities by in situ zymography and the cleavage of galectin-3 indicating that this differential staining could indeed be used as a surrogate diagnostic marker for MMP activity in the developing breast tumor.

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P55-3: CYTOMETRIC ANALYSIS OF MULTIPLEX ANTIGEN STAINING IN HISTOPATHOLOGY

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Histopathology plays an essential role in cancer diagnosis and classification, and antibody staining has introduced molecular analysis into histological studies. Currently, visual and computer-assisted image analysis of immunostained slides is based on area and intensity of staining. We describe a novel cell-based or cytometric approach to immunohistological analysis that uses multispectral microscopy to image multiplex-stained slides. Spectral unmixing software (Nuance[®]) is used to resolve the individual stains in images into separate channels. Image analysis software (FARSIGHT) is used to delineate individual nuclei using the DAPI or hematoxylin channel and associate analyte staining in the other channels with the segmented nuclei to assign and quantify antigen expression cytometrically. Methods have been developed to subtract background staining, and the resulting data can be displayed in a variety of formats for subsequent analysis. We provide examples of uniplex and multiplex staining in human breast cancer specimens immunostained for estrogen and progesterone receptors and for phospho-ERK and Ki67 antigens. We also used this method to examine the pharmacodynamics of breast cancer treatment with lapatinib. These examples illustrate the advantages of multiplex immunohistological studies and how histocytometry can be used to study molecular events and processes in human cancers, allows retrieval of more of the biological information that is preserved in fixed human tissues, and has the potential to become an effective tool for breast cancer translational research.

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P55-4: REDUCED EXPRESSION OF TOCOPHEROL-ASSOCIATED PROTEIN (TAP/SEC14L2) IN HUMAN BREAST CANCER

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The pathogenesis of breast cancer involves hormone-driven cell proliferation and the accumulation of gene mutations, including oncogene activation and suppressor gene downregulation. Although some genes, such as the Her2/neu oncogene and the p53 tumor suppressor gene, have been found to be overexpressed or mutated in about 20% of breast cancers, the predominant genes involved in the development of most of sporadic breast cancers remain to be elucidated. Tocopherol-associated protein, previously identified as tocopherol binding protein, has high homology to the yeast protein Sec14 and is also called Sec14L2 (TAP/Sec14L2). Our recent data suggest that TAP/Sec14L2 not only mediates vitamin E absorption, but it also functions like a tumor suppressor to control cell viability in prostate cancer. In this study, we show that TAP/Sec14L2 has a relatively and selectively high expression in nonmalignant breast, prostate, and liver tissues as compared to lung, colon, and kidney. Further analysis by quantitative PCR indicates that TAP expression is downregulated in MCF7 and MDA-MB-231 breast cancer cell lines. Immunohistochemical staining showed that 54% of 141 human invasive breast carcinomas had no TAP/Sec14L2 expression and that this increased to 88% in high-grade invasive carcinomas. This downregulation of TAP/Sec14L2 was also present in ductal carcinoma in situ (DCIS) associated with the invasive carcinoma. There was no association between TAP/Sec14L2 downregulation and patient age,

tumor size, or lymphovascular invasion/lymph node metastasis. These observations are consistent with the finding of deletion of chromosome 22q12 in several human carcinomas, including breast carcinoma. This study indicates that TAP/Sec14L2 is expressed by normal/benign breast epithelium and its downregulation in situ and invasive breast carcinomas raises the possibility that TAP/Sec14L2 may serve as a tumor suppressor-like factor in breast carcinogenesis.

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P55-5: INFLAMMATORY BIOMARKERS FOR EARLY DETECTION OF BREAST CANCER

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Background and Objective: Although recent advances in the early diagnosis of breast cancer have been beneficial, 34% of breast cancer patients have regional or distal spread of the disease at diagnosis [1]. This contributes to lower survival and suggests that further improvement in early diagnosis is urgently needed. Our objective is to discover nitrotyrosine (NT)-containing proteins serum biomarkers for early disease in patients with ductal adenocarcinoma of the breast.

Methodologies: We developed a number of methodologies including depletion of high abundance proteins (HAP) and immunoaffinity enrichment of NT-containing proteins. Coded patient sera were obtained from a prior study. A total of 100 μ L of serum diluted with 1:3 with bicarbonate buffer and was incubated with a 1 mL anti NT resin for 30 minutes. The resin was then washed extensively and bound proteins were eluted with 0.1 M glycine, pH 2.15. The eluted proteins were collected in 1.5 M Tris, pH 8.0 and acetone precipitated or separated on a 1D gel. Proteomics analysis was performed at the Proteomics Shared Facilities, Oregon Health & Science University.

Results to Date: We synthesized a 13 mer NT-containing peptide (YPDYDP-NT-AGGGK) and examined it by MS to identify its sequence. The peptide gives a strong signal by EMS and a strong y ion series in the MS/MS fragmentation patterns. In the sample preparation step, we eliminated the need to remove HAP since the NT affinity resin alone was sufficient to isolate NT-proteins and was found to be the only cleanup step required. To eliminate the problem of leached goat IgG upon elution, eluates will be subjected to protein G chromatography. Contained on a 1D gel of the purified serum in which antibody-related IgG was not removed, six proteins were found in the breast cancer sample that were not found in the control. However the data is only an n of 1 and needs to be repeated with an n >4 for statistical analysis. In addition, analysis of the remaining specimens will employ much more stringent sample-handling procedures designed to minimize the presence of keratin.

Conclusions: We have developed a unique approach to identify potential biomarkers of early breast cancer by enriching breast cancer patient's sera for nitrotyrosine, a product of nitrosative stress and marker of chronic inflammation, often in cancers [2]. Using this approach we isolated 6 NT-containing proteins unique to the serum of breast cancer patients. While other proteins were visualized, keratin contamination compromised their identification. However, we plan to examine the remaining specimens with stringent sample-handling techniques. The potential impact of this work is that it presents the physician the opportunity for more timely therapeutic intervention by providing a very early disease biomarker. This should result in improving long-term survival that is highly relevant to the goals of the Era of Hope.

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P55-6: DETECTION OF BREAST CANCER CELLS IN BLOOD BY QUANTIFICATION OF Her-2 GENE AMPLIFICATION IN SINGLE CELLS

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One of the early events in cancer metastasis is the shedding of cells from a primary tumor into the bloodstream. These cells, known as circulating tumor cells or CTCs, could be an important diagnostic and prognostic tool for early detection, therapy guidance, and recurrence monitoring. Though the importance of the detection and characterization of CTCs is beginning to be appreciated, their clinical utility has not been fully realized due to their low concentration in the bloodstream (1 CTC in 1×10^6 blood cells), making reliable detection difficult. Moreover, the current technologies in use to identify and characterize CTCs, such as RT-PCR and immunocytochemistry, are limited in the amount of information they can collect; neither is capable of both counting CTCs and characterizing multiple molecular markers at the same time.

We have developed a new multiplex fluorescent in situ hybridization (FISH) technology that has the potential to both enumerate CTCs as well as molecularly characterize multiple DNA and RNA markers. We have named this technology MAGEX for Multiplex Analysis of Gene Expression in Single Cells. The assay utilizes a probe set consisting of standard oligonucleotides to hybridize to a target gene of interest followed by signal amplification and fluorescent detection. Unlike standard FISH assays, the MAGEX probe set can span a target region less than a 1 kb and requires no direct labeling of the probe. Furthermore, the assay can be completed in less than a day and can be multiplexed.

The combination of novel probe design and signal amplification system simultaneously improves positive signals and reduces background, enabling highly sensitive detection of DNA and RNA markers in situ. Using the breast cancer cell line SK-BR-3, we have shown that the MAGEX assay has the ability to detect the Her2/Neu gene amplification. In the HeLa cell line, this gene amplification was not detected, but the normal two to four gene loci were easily identifiable. We have also demonstrated the ability of the MAGEX assay to detect mRNA. When Her2 gene expression was analyzed in HeLa and SK-BR-3 cells, there was a marked increase in the number of positive RNA spots in the SK-BR-3 cells. Interestingly, the sensitivity of the MAGEX assay is so high that single copies of mRNAs can be visualized as individual puncta. We have also shown for both RNA and DNA detection that multiple targets can be visualized at the same time. Furthermore, SK-BR-3 cells can be distinguished based on HER2 gene amplification when mixed with blood cells. These results represent a successful first step toward a MAGEX assay for CTC detection and characterization.

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P55-7: NOVEL APPROACH TO HARNESS THE POWER OF THE IMMUNE SYSTEM TO DISCOVER RARE, BLOOD-BORNE BREAST TUMOR MARKERS WITH DIAGNOSTIC POTENTIAL

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Fred Hutchinson Cancer Research Center

Diagnostic protein biomarkers are critical for minimizing death and suffering from breast cancer. For example, the estrogen receptor and Her2/neu status of a tumor are essential for choosing treatment regimens. Despite their potential to impact breast cancer through diagnosis, prognosis, guiding treatment decisions, and monitoring response to therapy, we have precious few biomarkers in clinical use.

Ideally, biomarkers would be secreted or shed by tumors yet be noninvasively measured in the bloodstream. Unfortunately, even with powerful technology such as mass spectrometry, the probability of discovering rare tumor-derived proteins in the presence of highly abundant normal plasma proteins is dismally small.

We attempted an innovative immune-based approach to discovering human breast cancer-derived proteins in circulation. We hypothesized that an immunocompetent mouse immunized with plasma from a congenic mouse harboring a human breast cancer xenograft would generate antibodies to circulating human breast cancer proteins of diagnostic value. In other words, we attempted to exploit the mouse immune system to detect only those human breast cancer-derived proteins present in the bloodstream by testing whether plasma from a xenografted mouse could be used to immunize a second congenic mouse against circulating human breast tumor antigens of diagnostic value. (Because the donor and recipient mice are congenic, only human proteins found in the donor plasma will be antigenic.)

Three immunodeficient NOD/SCID mice each were xenografted at the mammary fat pad with a human breast cancer cell line (MDA-MB-231) and tumors developed to ≥ 1 cm³. After tumor development, blood was harvested, and plasma was separated and depleted of the three most abundant proteins by affinity chromatography. Depleted plasma from each xenografted mouse was then both analyzed by mass spectrometry (which was able to detect human proteins in the mouse plasma) and also used to immunize three congenic, immunocompetent mice. Four immunizations were given subcutaneously to each mouse over a period of 42 days. Plasma was harvested from the immunized mice, and immunoblotting was performed against a variety of lysates prepared from MDA-MB-231 cells to screen for reactivity to human proteins. Pre- and postimmunization western blots were compared for each type of cell lysate, and differences were noted. Two classes of changes were observed in the immunoreactivity of pre-versus postimmune serum: (1) some proteins detected in the preimmune blots appeared to be more intense in the postimmune blots, likely representing waxing and waning titers not specific to the tumors and (2) some proteins detected in the postimmune blots were not detected in preimmune blots, suggesting that immunoreactivity to these proteins had been stimulated by circulating proteins emanating from the xenograft. We conclude that human proteins enter the circulation in mice xenografted with MDA-MB-231 cells and that plasma from xenografted mice can be used to successfully stimulate a congenic immunocompetent mouse to generate an immune response to circulating human antigens.

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P55-8: EARLY DETECTION OF BREAST CANCER

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Improved early detection of breast cancer will have the most dramatic positive impact on breast cancer survival. Population-wide prevention is not readily available for this disease because there are no simple life style changes known that will markedly reduce the incidence of breast cancer. Thus, earlier detection will improve therapeutic success of this deadly disease because when the cancer is caught before metastases occur, it is still curable by surgery. A large number of laboratories and companies are therefore pursuing detection of breast cancer-associated proteins and protein profiles, or signatures, in serum from breast cancer patients. While such proteins have been successfully identified and are being used clinically for early detection of prostate cancer, progress in serum biomarkers for breast cancer has been very slow. In fact, there are no accepted clinically useful serum biomarker proteins for early detection of human breast cancer.

We applied a novel strategy to identify early breast cancer biomarkers that can be induced to detectable levels. Specifically, we searched for secretory gene products that are induced by prolactin. First, we identified candidate secretory genes using genome-wide transcript analysis of human breast tumor lines in response to prolactin and performed systematic validation to demonstrate their utility. Second, selected genes were validated in the more physiologically relevant xenotransplant mouse model, and measurements were done on inducible biomarker levels in serum. We have examined whether the candidate genes are induced in human breast cancer tissues that are grown in tissue culture in the presence or absence of prolactin. Both gene expression and protein secretion from cells into the media or serum were studied to validate the utility of these candidate biomarker genes. From a 33,000 human gene expression array, we have identified and confirmed 9 secretory genes that have increased levels of RNA expression in prolactin-treated breast cancer cells.

A benefit of our new approach is that one could identify tumor-associated biomarker and rapidly set up sensitive immuno-based detection assays for use in the clinic. A practical test that involves blood sampling of patients at high risk of breast cancer may be developed based on these data. Improved early detection of breast cancer before metastases occur will have the most significant impact on the ACS programmatic goal of reducing death and suffering from breast cancer.

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P55-9: ATYPIA AND DNA METHYLATION IN NIPPLE DUCT LAVAGE IN RELATION TO PREDICTED BREAST CANCER RISK

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Background: Tumor suppressor gene (TSG) methylation is identified more frequently in random periareolar fine needle aspiration samples from women at high risk for breast cancer than women at lower risk. It is not known whether TSG methylation or atypia in nipple duct lavage (NDL) samples is related to predicted breast cancer risk.

Methods: A total of 514 NDL samples obtained from 150 women selected to represent a wide range of breast cancer risk were evaluated cytologically and by quantitative multiplex methylation-specific PCR for methylation of cyclin D2, APC, HIN1, RASSF1A, and RAR-b2.

Results: Based on methylation patterns and cytology, NDL retrieved cancer cells from only 9% breasts ipsilateral to a breast cancer. Methylation of ≥ 2 genes correlated with marked atypia by univariate analysis but not multivariate analysis that adjusted for sample cellularity and risk group classification. Both marked atypia and TSG methylation independently predicted abundant cellularity in multivariate analyses. Discrimination between Gail lower risk ducts and Gail high-risk ducts was similar for marked atypia (O.R. = 3.48, P = 0.06) and measures of TSG methylation (O.R. = 3.51, P = 0.03). However, marked atypia provided better discrimination between Gail lower risk ducts and ducts contralateral to a breast cancer (O.R. = 6.91, P = 0.003 compared to methylation (O.R. = 4.21, P = 0.02).

Conclusions: TSG methylation in NDL samples does not predict marked atypia after correcting for sample cellularity and risk group classification. Rather, both methylation and marked atypia are independently associated with highly cellular samples, Gail model risk classifications, and a personal history of breast cancer. This suggests the existence of related, but independent, pathogenic pathways in breast epithelium.

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P55-10: TELOMERE DNA CONTENT PREDICTS OVERALL AND BREAST CANCER-FREE SURVIVAL INTERVALS

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Background: There is a pressing need for new markers that accurately predict the likelihood of breast cancer recurrence. Telomeres are nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination. Critically shortened telomeres generate genomic instability. It has been postulated that the extent of telomere DNA loss is related to the degree of genomic instability within a tumor, and therefore may presage clinical outcome. The objective of this investigation was to evaluate the hypothesis that telomere DNA content (TC), a proxy for telomere length, in breast tumor tissues predicts overall and breast cancer-free survival intervals.

Methods: Slot blot titration assay was used to quantitate TC in archival breast tumor tissues from 530 members of the New Mexico subset of the NCI/SEER Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort. The relationships between TC and 12 risk factors for breast cancer adverse events (death due to breast cancer, breast cancer recurrence, or new primary breast tumor) were evaluated by Fisher's Exact Test. The relationships between TC, overall survival interval, and breast cancer-free survival interval were evaluated by log-rank analyses and displayed by Kaplan-Meier survival plots. Multivariate Cox proportional hazards models were used to evaluate the relationships between TC and 12 risk factors for breast cancer-free survival interval.

Results: In all tissues, log-rank analysis showed a significant relationship between TC group, low TC ($\leq 200\%$ of standard) and high TC ($> 200\%$ of standard), and overall survival (p=0.025) and breast cancer-free survival (p=0.009) intervals. Additional analysis in the subset of invasive tumors (n=433) showed a significant relationship between TC group and overall survival (p=0.046) and breast cancer-free survival (p=0.032) intervals. Similar, although not statistically significant, results were shown in the subset of DCIS cases (n=97). The best overall multivariate Cox proportional hazards model included TC, p53 status, TNM stage, and ER status as independent predictors of breast cancer-free survival interval (p<0.00005). Low TC, relative to the high TC group, conferred an adjusted relative hazard of 2.88 (95% CI=1.16-7.15; p=0.022) for breast cancer-related adverse events.

Conclusions: TC in breast cancer tissue is an independent predictor of overall and breast cancer-free survival intervals. In the future, TC in combination with extant prognostic markers could provide women and their physician's new information to guide therapeutic decisions.

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P55-11: BLOOD TUMOR MARKERS FOR MOLECULAR DIAGNOSIS OF BREAST CANCER: CORRELATION WITH DISEASE COURSE

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Introduction: Breast cancer is the most common cancer occurring in women, accounting for a third of all female cancers. Death from breast cancer is usually due to metastatic spread to other organs, which can occur hematogenously. Our objective was to establish a specimen bank of serial prospectively collected clinically annotated peripheral blood specimens to test the clinical utility of early detection technologies in breast cancer management. The primary assay to be tested was a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assay targeted to a molecule called keratin-19, or *K19*. It is believed that a detectable level of *K19* mRNA signifies the presence of circulating tumor cells (CTC) and that trends in levels may predict tumor burden and/or prognosis.

Methods: Patients scheduled for diagnostic breast biopsy are approached for enrollment into a prospective cohort study. Blood is drawn prior to biopsy, and if diagnostic work-up reveals invasive breast cancer, they are followed with serial blood draws and clinical data collection every 6 months. If they are found to have Stage IV disease, follow-up is performed every 6 weeks, and a separate tube is also drawn for the commercially available Veridex CellSearch™ CTC assay. Monthly *K19* levels measured by qRT-PCR in patients with metastatic disease are compared to objective tumor response. Similar analyses will be performed with the CellSearch™ assay for a comparison of methods. At this time data is available on the metastatic cohort for the *K19* qRT-PCR assay. The dynamic range of this assay was found to extend to a detection limit of 0.010fg.

Results: A total of 153 subjects have enrolled in the study to date. The enrolled subjects have a full spectrum of breast disease, including 31 benign, 25 carcinoma in situ, and 97 invasive breast cancer. Twenty subjects had metastatic disease at the time of enrollment, and they have been followed for a median of 15.4 months thus far. Eleven of 20 subjects had detectable levels of *K19* at the time of enrollment. Subjects with detectable *K19* mRNA at baseline were 5.7 times more likely to progress on therapy ($p=0.0281$) and the level of *K19* correlated with outcome. Seven of the 11 subjects with detectable *K19* were observed to progress on therapy (64%), and they had a mean level of 0.705fg prior to initiation of treatment. Three subjects with detectable *K19* were observed to have stable disease (27%), with a mean baseline *K19* level of 0.532fg. Only 1 subject with detectable *K19* was observed to respond to treatment (9%), but that level was barely detectable at 0.012fg. Longitudinal models are currently being constructed to evaluate the trends in *K19* mRNA levels observed over serial blood collections and their relationship to disease outcome.

Conclusions: Recruitment into a clinically annotated serial peripheral blood specimen bank from a prospectively followed cohort of women with a spectrum of breast disease is feasible and useful for collaborative research. *K19* mRNA levels detected in the peripheral blood of women with metastatic breast cancer may be useful to predict a poor response to therapy. Future directions include expansion of the *K19* qRT-PCR assay to incorporate additional tumor markers that may help determine alternate therapies that may be more useful for women with refractory metastatic disease.

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P55-12: RECOMBINANT PEPTIDES AS BIOMARKERS FOR METASTATIC BREAST CANCER RESPONSE TO MOLECULAR-TARGETED THERAPY

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Objective: Presently, metastatic breast cancer response is measured by assessment of tumor volumes or by repeated biopsy to analyze pharmacodynamics. These methods of monitoring breast cancer response are inefficient because volume changes typically occur after patients are on therapy for prolonged time intervals. For that reason, we have developed the hypotheses that recombinant peptides from phage-displayed peptide libraries can be selected that bind to receptors activated in response to therapy with tyrosine kinase inhibitors (TKIs). These peptides in turn can be labeled with internal emitters to provide a means to noninvasive monitoring of responsiveness to therapy. The physiologic response to therapy is seen within 24 hours of therapy, which provides a rapid assessment using noninvasive means.

Materials and Methods: In vivo biopanning was conducted with a T7 phage-based random peptide library on MDA-MB-231 and MCF-7 tumors treated with Sunitinib. Four rounds of consecutive selections were performed, and phage clones were identified by sequencing the genomic DNA fragments encoding the corresponding peptides. Peptides were labeled with Alexa-Fluor 750 to provide a means of noninvasive monitoring of cancer responsiveness to therapy and quantify the effectiveness of therapy utilizing near-infrared imaging. By employing MDA cell lines that have been stably transfected with either luciferase or GFP, we examined the biological effects of peptide binding to tumor by tumor size measurement and intensity of luciferase or GFP signal in treated nude mice. Histological analyses of the tumors were performed to assess peptide binding.

Results: Sunitinib treatment elicits growth delay in nude mice implanted with MDA-MB-231 breast tumors. Sequence analysis of phage recovered from in vivo biopanning of MDA cells showed the following distribution: EGEVGLG, SSAVL, MRRSVGS, FGVR, VLI, SAGSVAL, and GFWEGL. The first four peptides were also isolated from in vivo biopanning of MCF-7 cells. Imaging studies of those peptides indicate that they preferentially bind to Sunitinib-treated MDA tumors when compared to untreated controls as the intensity of peptide binding correlates with a decrease in tumor size and decrease in luciferase or GFP activity. Immunohistochemical analysis shows that the peptide binds to treated tumor endothelium.

Conclusions: We have identified recombinant peptides isolated from breast tumors that discern responding from nonresponding breast tumors after TKI treatment. Since the peptides can bind to responding breast cancer cells that have luciferase or GFP stably transfected MDA cells, we have developed a model that can detect treatment response in metastatic breast tumors. This is platform technology that shows the principle that recombinant biomarkers are effective at rapidly reassessing breast cancer susceptibility to molecular-targeted therapy.

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P55-13: EVALUATION OF BREAST-TISSUE ESTRADIOL AND TRIGLYCERIDES FROM A HIGH-RISK COHORT

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Using patient-matched serum and extracellular fluid from random periareolar fine needle aspiration samples (RPFNA-EF), obtained from women at high-risk for developing breast cancer, we evaluated estradiol (E2) and triglyceride (TG) levels. We compared these values to the menopausal status, BMI, and Masood cytology index values for each patient. In general, the estradiol levels of the RPFNA-EF compartment were 3X that observed in the serum. There were no statistical differences in the tissue level estradiol concentrations based on menopausal status. There was a slight, but significant, negative correlation between BMI and RPFNA estradiol/TG levels. Tissue level estradiol levels were associated with increasing Masood cytology index.

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P55-14: THE BIOLOGICAL FUNCTION ANALYSIS OF DELTA-N-p63 IN BREAST CELLS

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There has been abundant evidence that the p53 family member TP63 plays a critical role in making decision to preserve or forfeit mammary progenitor cells' self-renewing capacity. Our previous study clearly demonstrated that ectopic delta-N-p63 (δN -p63), the N-terminally deleted isoform of TP63, could block retinoic acid-induced differentiation in embryonic carcinoma cells NT2/D1 and preserve transcript level of nestin post RA treatment, which is a regenerative cellular marker in nervous system and embryo development. The biological function of δN -p63 in breast cancer cell lines and its correlations with other well-known cellular self-renewal relative genes such as Oct 3/4, Nanog were further investigated. Similarly, RA treatment could inhibit the proliferation of breast cancer cell lines including MCF-7, MDA-MB-231, and SUM102, and downregulate the mRNA level of some self-renewing relative genes including Oct 3/4, Nanog in these cell lines. Immunocytofluorescence staining detected existence of δN -p63 protein in both estrogen receptor-negative cells such as SUM102 and MDA-MB-231 cells but also MCF-7 cells with luminal epithelial phenotype. Interestingly, immunocytofluorescence double staining displayed that δN -p63-positive cells in MCF-7 are not well differentiated and lost expression of cell cycle marker ki-67, cyclin D1. Infection of breast cancer cells with δN -p63 adenovirus could decrease cell growth rate by cell counting assay and cause G1/G0 cell phase arrest showed with PI staining flowcytometry. In MCF-7 cells, ectopic δN -p63 could induce cells to lose expression of ki-67 and cyclin D1. Quantitative PCR assay showed that over expression of δN -p63 could up-regulate transcript level of Oct3/4 and Nanog in MCF-7 cells. CD24/CD29 were applied to isolate stem cells (p6) and progenitor cells (p5) from normal and PATCH (-/+) mice breast tissue, respectively. RT-PCR assay detected the transcript of Oct3/4 and Nanog in both p5 and p6 populations. Further, quantitative PCR analysis demonstrated that the transcript level of Oct3/4 in mice stem cell was higher compared to progenitor cell. More importantly, the overexpression of ectopic δN -p63 in progenitor cells and knock down of endogenous δN -p63 in stem cells could increase and decrease Oct3/4 transcript level, respectively. In contrast, there was the reverse correlation between Nanog transcript level and expression level of δN -p63 in both mice stem cell and progenitor cell subsets. These data implicated that δN -p63 and other embryonic stem cell marker genes could collaborate to play a role in the regulation process of breast cell regenerations.

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P55-15: DNA HYPERMETHYLATION PATTERNS DETECTED IN SERUM AS A TOOL FOR EARLY BREAST CANCER DIAGNOSIS

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Studies in breast and other cancers have shown that the promoter regions of some genes, in particular tumor suppressor genes, are often hypermethylated in cancer but not normal cells and that this methylation may be an early event in carcinogenesis. Through necrosis and apoptosis, tumors release genomic DNA into the systemic circulation. Analysis of this DNA found in the serum/plasma of breast cancer cases allows for the detection of promoter hypermethylation with results showing good concordance with paired tumor tissue samples. This study examines the potential of serum DNA hypermethylation markers as tools for the early detection of breast cancer. To date, no other study has used serum collected prior to breast cancer diagnosis. This is a case-control study nested within the NYU Women's Health Study (NYU WHS) cohort. The NYU WHS enrolled 14,274 healthy women ages 35–65 between the years 1985 and 1991. Serum was collected from each participant and stored for future biochemical analyses. To date, using active follow-up as well as linkages to tumor registries, a total

of 1,006 incident invasive breast cancer cases have been diagnosed. In this study, cases are those women for whom we have a blood sample collected within the 6 months preceding breast cancer diagnosis (n=113). Assays are in progress for the first 50 cases whose mean age at diagnosis was 53.1 years. Forty percent of the tumors were diagnosed at stage 1, 34% at stage 2A, 6% at stage 2B, and 2% at stage 3A. Tumor stage was unknown for 9 cases (18%). Each case was assigned two controls without and one control with a history of benign breast disease. These controls were selected from among women who were alive and free of any cancer at the date of case diagnosis. These two control groups will be used to assess the ability of the methylation markers to distinguish between normal and malignant tissue (healthy controls) and between benign and malignant breast disease (benign breast disease controls). Controls are matched to the case on age at, and date of, blood donation to control for the potential effects of age on methylation and the length of sample storage time. The promoter methylation status of a panel of 6 cancer-related genes (RASSF1A, GSTP1, RARβ2, APC, HIN-1, and CDKN2A [p16]) is being assessed using quantitative real-time MSP methods. Early breast cancer diagnosis is a significant determinant of overall breast cancer survival. This study seeks to determine whether promoter methylation status of tumor suppressor genes, detected in serum, may be used as a marker for the early detection of breast cancer.

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P55-16: HOW SPECIFIC FOR BREAST CANCER ARE SERUM BIOMARKERS IN PREMENOPAUSAL WOMEN?

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Background and Objectives: Although serum biomarkers have had success as diagnostic aids for some cancers such as ovarian cancer and prostate cancer, their use for breast cancer is still in question. Their weaker diagnostic ability suggests that they may measure the more general secondary effects of breast cancer rather than specifically the cancer itself.

This study focuses on premenopausal women who have increased breast density and therefore benefit less from breast cancer screening by common imaging technologies. To complement imaging-based screening for breast cancer in premenopausal women, serum biomarkers are tested for their diagnostic ability and specificity for breast cancer.

Methods: This study used a set of 98 serum proteins and chose diagnostically relevant subsets via various feature-selection techniques. Because of significant noise in the data set, we applied iterated Bayesian model averaging to account for model selection uncertainty and to improve generalization performance. The Bayesian approach also allowed for comparing the posterior distributions of each biomarker for the benign, malignant lesions and normal breast tissue. We assessed generalization performance using leave-one-out cross-validation and receiver operating characteristic (ROC) curve analysis. Bayesian model averaging was compared to other classification algorithms.

Results: The classifiers were able to distinguish normal tissue from breast cancer with a classification performance of $AUC=0.82\pm0.04$ with the proteins MIF, MMP-9, and MPO. The classifiers separated normal tissue from benign lesions similarly at $AUC=0.80\pm0.05$. However, the serum proteins of benign and malignant lesions were indistinguishable ($AUC=0.55\pm0.06$). The classification tasks of normal versus cancer and normal versus benign selected the same top feature, MIF, which suggests inflammatory response.

Conclusions: Overall, the selected serum proteins showed promise in detecting breast lesions (benign or malignant) but are probably more indicative of secondary effects rather than specific for malignancy. Therefore, scientists should continue to search for serum biomarkers that are more specific for breast cancer.

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P55-17: IDENTIFICATION OF PREGNANCY-ASSOCIATED BREAST CANCER (PABC) FOR INVESTIGATION OF THE ROLE OF MAMMARY GLAND INVOLUTION IN PROMOTING METASTASIS IN A NORWEGIAN COHORT OF BREAST CANCER CASES

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Full-term pregnancy increases breast cancer risk for up to 10 years after pregnancy and breast cancer diagnosed during this time has been referred to as PABC (Albrektsen, *Br. J. Cancer*, 2005). Importantly, PABC patients have high rates of metastasis, which is independent of known prognostic factors. Following parturition or lactation, during involution, the breast returns to its pre-pregnant state. In a rat model, we found mammary gland involution employs tissue remodeling programs activated during wound healing and inflammation including high matrix metalloproteinase -2, -3, and -9 activities, release of bioactive fragments of FN and LN, deposition of fibrillar collagen, and increased cytokine levels, including TGFβ1, TGFβ3, and connective tissue growth factor (McDaniel, *AJP*, 2006, & Schedin, *JMGB&N*, 2007). We hypothesize that this physiologically normal, but pro-inflammatory remodeling of the gland accounts for the high rate of metastases seen in PABC (Schedin, *Nature Reviews Cancer*, 2007).

To pursue this investigation in human breast cancer, we are utilizing a resource of an extensive cohort of Norwegian women diagnosed with breast cancer. These cases have been cross-referenced with national birth records to accurately identify parity status and time of parity in relation to breast cancer diagnosis. A total of 20,192 cases of breast cancer are in the cohort, of which 3,044 were under the age of 40 at time of breast cancer diagnosis. Focusing on women in the under-age-40 cohort, 500 were nulliparous and 2,544 had prior completed childbirth. Five hundred sixty-four were uniparous, 1,350 biparous, and 630 triparous. Women with higher parity numbers were not included in the cohort. The true duration of risk for increased metastatic potential from breast cancers diagnosed after pregnancy has not been clearly defined. This increased risk of systemic recurrence may last up to 6 years or more after a completed birth. From the Norwegian cohort, 45% of women diagnosed with breast cancer under age 40 would meet the definition of PABC. A more conservative approach of a 2-year time definition still demonstrates 18% of women in this cohort meet the PABC definition, highlighting the large number of women at risk for increased metastatic spread of their cancer due to PABC. Women whose cancer was diagnosed before age 40 had an average age at diagnosis of 31.8 if PABC and 34.7 if non-PABC (nulliparous), suggesting a promotional effect of the pregnancy on the cancer.

From this cohort of 3,044 women diagnosed under age 40, we will retrieve paraffin-embedded tumor blocks on 100 cases of PABC and 100 cases of non-PABC for analysis of pro-inflammatory markers. This analysis will include immune cell markers CD45 and CD68, the oncofetal ECM protein tenascin, matrix metalloproteinase -2 and -9, fibronectin, and other markers. The identified PABC signature will be correlated with clinical outcomes. We predict that PABC, having arisen in the pro-inflammatory microenvironment of the involuting gland, will be characterized by a reactive stroma.

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STEM CELLS II

Poster Session P56

P56-1: NUMB-MEDIATED ASYMMETRIC CELL DIVISION IN MAMMARY GLAND DEVELOPMENT

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The apparent similarity between the ability of cancer cells to grow uncontrollably and the most prominent property of stem cells, namely their ability to continuously self-renew, has led to the notion that cancers are diseases of stem cells. It is believed that only a small fraction of the cells within the tumor mass is capable of generating tumors. We are using mammalian Numb proteins as an entry point to examine the importance of two modes cell division—symmetric versus asymmetric—in allowing stem cells to balance the competing needs of self-renewal and differentiation during mouse neurogenesis and mammary gland development, as a means to examine the consequence of altering the self-renewing capability of stem cells in organogenesis and tumorigenesis.

Asymmetric cell division is a process by which a cell divides to produce two different daughter cells. Conceptually, such divisions are an attractive means for stem cells to balance the needs of self-renewal and differentiation during organogenesis and tissue maintenance, by producing one daughter cell that self-renews and another that differentiates. Studies using invertebrates show that intrinsically asymmetric cell divisions are made possible by a complex, but evolutionarily conserved, molecular machinery that polarizes cells and subsequently enables cell-fate determinants to be differentially inherited by the two daughter cells, thereby allowing them to adopt different fates. A key determinant of asymmetric cell fates in *Drosophila* is Numb, a cytoplasmic signaling protein.

As a means to determine whether Numb-mediated asymmetric cell division is a mechanism shared by stem cells in many tissues for their progeny to choose between self-renewal and differentiation, we have been performing parallel experiments in the developing nervous system and mammary gland to examine the roles played by the mammalian Numb homologues. Our findings show that the two mouse numb genes, *numb* (Numb) and *numbl* (Numbl), are functionally redundant and that asymmetric segregation of the Numb proteins and, therefore, asymmetric cell division are essential for stem/progenitor cells to balance self-renewal and differentiation. We further show that stem-cell numbers are strictly controlled in vivo and that tumor suppressors likely play a key role in maintaining stem-cell numbers homeostasis.

A fundamental issue in cancer biology and treatment is why killing the vast majority of cancer cells does not lead to a cure. The notion of cancer stem cells is attractive in this regard because stem cells have limitless self-renewal capability and, therefore, even a few cells may be sufficient to form new tumors and, consequently, cause relapse and prevent cure. We propose that ways to manipulate the division pattern of stem cells may point to fundamentally different targets for devising therapeutic measures to treat cancer.

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P56-2: EPIGENETIC REGULATION OF HUMAN NORMAL MAMMARY EPITHELIAL STEM CELL FUNCTION AND DIFFERENTIATION

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The existence of stem cells in the normal human mammary epithelium has been postulated, but the cellular identities and molecular characteristics of these cells have not been defined. Similarly, the high degree of heterogeneity of breast carcinomas has been proposed to be due to the progenitor-like properties of tumor-initiating cells. The normal functioning and differentiation of stem cells are regulated by epigenetic programs, and alterations in these may play a role in tumorigenesis. To address these related issues, we purified four phenotypically distinct mammary epithelial progenitor and differentiated cell populations from normal human breast tissue and determined their comprehensive gene expression and DNA methylation profiles and in vitro differentiation capacity. We identified DNA methylation patterns correlating with cell type and differentiation state that were maintained in breast carcinomas and classified them into clinically relevant subtypes. The expression of FOXC1, a transcription factor hypomethylated and highly expressed in our purified CD44+ stem cells, induced a progenitor-like phenotype in differentiated epithelial cells. These findings highlight the importance of cell type-specific epigenetic programs in determining cellular phenotypes and have implications for understanding the origin and differentiation capacity of breast carcinomas.

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P56-3: DYSREGULATED Wnt SIGNALING IN DMBA-INDUCED MOUSE MAMMARY TUMORS SUGGESTS A STEM CELL ORIGIN

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Mammary tumors induced in mice by oral administration of the carcinogen dimethylbenzanthracene (DMBA) are histologically diverse. Using antibodies to cytokeratin 14 (CK14), which is expressed in myoepithelial cells, and to cytokeratin 18 (CK18), expressed in luminal epithelial cells, we correlated this histologic diversity with differences in the relative proportion of CK14 and CK18 expressing cells in individual tumors. This observation suggests that these tumors arise from a progenitor or stem cell that has the ability to differentiate along multiple pathways. We noticed that the DMBA tumors were phenotypically similar to tumors induced in mice by dysregulation of different components of the Wnt signaling pathway. Thus, we asked if Wnt signaling, which is important for mammary stem cell function, is dysregulated in the DMBA-induced tumors as well. We assessed the localization of beta-catenin in these tumors since nuclear localization of this protein is a downstream consequence of upregulated signaling through the canonical Wnt pathway. We found that beta-catenin was localized to the nucleus of a subset of cells in all (n=9) DMBA-induced tumors examined. Strikingly, nuclear beta-catenin was found almost exclusively in CK14 positive cells. Cyclin D, which is a downstream target of Wnt signaling, was also more highly expressed in CK14 positive cells. In addition, expression of axin-2, a common target of Wnt/beta-catenin signaling, is more highly expressed in DMBA-induced tumors than in myc/ras tumors. These results confirm the upregulation of the Wnt/beta-catenin pathway in the DMBA tumors. In contrast to nuclear beta-catenin, cell proliferation as measured by BrdU incorporation was more extensive in the CK18 positive population than in the CK14 cells. These results suggest that there may be crosstalk between these cell types in promoting tumor growth. Accordingly, when tumor cells were dissociated into single cell suspensions and injected into the mammary fat pad of syngeneic mice, the phenotype of the parent and transplanted tumor were very similar. Together, these results suggest that DMBA targets the canonical Wnt signaling pathway in a mammary stem cell and that this mutated stem cell differentiates into tumor cells with characteristics of the two mammary gland lineages during tumor development. Furthermore, we suggest that these two cell types are both required for tumor growth. These results showing that the Wnt signaling pathway is targeted by a chemical carcinogen in the mouse mammary gland may have important implications for environmental carcinogenesis in humans.

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P56-4: SCRIBBLE LOSS ENRICHES FOR A MAMMARY PROGENITOR CELL

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There is increasing evidence to suggest that cells with pluripotent/stem cell-like properties are the drivers of cancers in humans. Molecular events that control the maintenance of cells in an undifferentiated, pluripotent state are likely to increase susceptibility to cancer. This is exemplified by changes in the Notch and Hedgehog signaling pathways that are implicated in specification, maintenance, and activation of stem cells and are also implicated in initiation of carcinoma. Interestingly, studies in model organisms such as *Drosophila* suggest a role for regulators of cell polarity in controlling cell fate decisions during neurogenesis. In *Drosophila*, loss of polarity genes in the Scribble/Lgl/Dlg complex inhibits neuroblast differentiation and increases the number of neuroblast progenitors during neurogenesis. Whether cell polarity pathways regulate differentiation of pluripotent cells in mammals is not known.

To investigate the role of Scribble loss, we downregulated Scribble with stable shRNAs in a pluripotent mammary epithelial cell line, Comma-1D. Loss of expression of Scribble induced a two- to three-fold expansion of cells expressing the basal markers (cytokeratins 5/6/14) and a concomitant decrease in cells expressing differentiated epithelial markers (cytokeratin 18 and E-cadherin). To understand the effect of this shift in cell populations on progenitor capacity, we used a mammosphere culture assay and observed that cells with reduced Scribble levels have an increased mammosphere-forming potential, indicating their increased ability to maintain a pluripotent state. Additionally, limiting dilution experiments in the cleared fat pad demonstrate a 25% increase in repopulation efficiency of scribble knockdown cells compared to controls. Furthermore, downregulation of Scribble increased the number of cells expressing aldehyde dehydrogenase, a marker for progenitor/tumor initiating cells, in a human breast cancer-derived cell line and when transplanted into nude mice, tumors grew at a twofold higher rate than control cells.

Our results implicate the polarity gene Scribble in the regulation of mammary epithelial cell differentiation. Thus we establish a role for polarity signaling pathways during differentiation of mammary epithelial cells.

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P56-5: IDENTIFICATION AND TARGETED THERAPY FOR BREAST CANCER INITIATING CELLS

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There is growing evidence that cancer may be organized in a hierarchy in which only a fraction of immortal cells, termed Tumor Initiating Cells (TICs; also referred to as Cancer Stem Cells, CSC), is capable of maintaining and propagating cancer and metastatic disease. In contrast, the majority of tumor cells represents mortal progenitor and partially differentiated cells that have lost their tumorigenic potential. Thus, targeted killing of TICs may be curative. Our laboratory is using several mouse models for specific breast cancer subtypes to analyze TICs, their biology, the pathways by which they divide and their unique properties relative to mammary stem cells. We recently reported on the first identification of TICs in a mouse model of Her2/Neu (Liu et al., 2007 *Cancer Res.*). Lineage-depleted tumor epithelial cells were sorted on the basis of cell surface marker expression and transplanted into the mammary gland of isogenic mice. Her2/Neu TIC cells were identified in the CD24⁺:Sca1⁻ fraction. This cell fraction also contains tumorsphere-initiating cells, which give rise to spheres in non-adherent conditions in serum-free medium containing FGF and EGF. By several criteria, the Her2/Neu TICs are indistinguishable from tumorsphere-initiating cells, hence providing a means by which to identify TIC-specific therapeutic targets by screening small molecule libraries for inhibitors of tumorsphere formation. Progress in the identification of TICs from mouse models of basal breast cancer (Pten and Rb) will also be presented (Liu et al., in progress; Jiang et al., in preparation). Our long-term goals are to use these approaches to develop TIC-specific inhibitors for the major breast cancer subtypes. Finally, we have tested the ability of a candidate breast cancer therapeutic drug to target TICs. We found that this drug preferentially kills CD24^{low}/CD44⁺ TICs from human pleural effusions, MCF7 cells as well as TICs from three different mouse models of breast cancer (Deng et al., in preparation).

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P56-6: THE "BIG BANG" OF BREAST ONCOLOGY: THE ORIGIN OF MAMMARY TUMORS

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It is now widely believed that the targets of carcinogens are stem cells in the breast. However, such cells are not well characterized and may actually represent a hierarchical range from lineage-committed to uncommitted stem cells, like bone marrow-derived multipotent adult progenitor cells, which can be recruited during breast development. We aim to test if bone marrow-derived stem cells can give rise to mammary tumors in a radiation-induced mouse mammary tumor model. To this end, p53 knockout mice on a BALB/c background, which is critical to the aims of this proposed project, had to be recovered from cryopreserved embryos at Jackson Laboratories. Following a lengthy process that included animal recovery for establishment of founders, verification testing, shipment, and quarantine, breeding pairs were entered into our defined flora, pathogen-free facility. To date, we have generated 58 mice (34 males, 24 females). Genotyping of these animals shows that 11 are p53^{-/-} (6 male, 5 female). Due to the short lifespan of these mice (approx. 3–6 months), production of homozygote knockouts to be used in our study has been slower than anticipated. As an alternative, we are considering using homozygote pups from heterozygote parents and using them for the study rather than for breeding.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0532.

P56-7: THE ISOLATION AND CHARACTERIZATION OF MOUSE MAMMARY STEM CELLS

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Background/Objectives: Prior studies show that cancers are heterogeneous with respect to the cells that are tumorigenic. This observation supports the cancer stem cell (CSC) hypothesis. CSCs may arise via tumorigenic mutations in tissue stem cells (TSC) or by mutations in progenitor cells that enable them to self-renew and form tumors. Like some TSCs, CSCs may divide infrequently and export drugs rapidly, engendering resistance to conventional therapies that target dividing cells. This may explain why tumor regression does not always translate into increased patient survival. Importantly, neither mammary CSCs nor mammary stem cells (MSCs) have been unambiguously identified. Therefore, the goal of this project is to develop functional genetic strategies that enable the isolation and characterization of mouse MSCs and to determine their relationship to CSCs.

Functional Genetic Strategy: One characteristic of some TSCs is that they divide infrequently and hence retain DNA-specific labels. We therefore developed a genetic strategy in mice using fluorescent histones (H2B-GFP) to label DNA. The K14 promoter or a Wnt-responsive promoter was used to direct the expression of H2B-GFP in the mammary, and a doxycycline (dox)-regulated transactivator was used to temporally control H2B-GFP labeling. By adding dox to the mouse diet at specific times, we could pulse and chase the cells in vivo and determine whether any retained H2B-GFP fluorescence. Labeled cells were then isolated in a micro-scale cell sorter and transplanted into de-epithelialized fat pads to determine their ability to regenerate mammary glands. In parallel, our focus has been on the analysis of early embryonic mammary structures to test the hypothesis that they are enriched for MSCs at stages prior to overt mammary differentiation.

Preliminary Results: We found that intact mammary rudiments give 100% repopulation efficiency. Given the limited cell number in such structures, the data suggest a significant enrichment of MSCs relative to the concentration in adult glands. Limiting dilution analyses are under way to quantify their abundance. We have also validated the use of microfluidics-based MSC purification using the K14 promoter and H2B-GFP labeling and are currently using these purification strategies to obtain gene expression profiles.

We have also developed a recombinase-based strategy to introduce single copies of mammary-relevant promoters into predetermined open loci. This strategy showed faithful Wnt induction, which is difficult to achieve with conventional transgenics.

Impact: Conventional cancer therapies may fail if they do not target the relevant cell type, such as putative CSCs. If CSCs initiate tumor formation and contribute to drug resistance, their characterization might enable earlier detection, more accurate prognoses, and the development of curative therapies. Our novel genetic and functional approaches should enable us to isolate and characterize MSCs and determine whether they or transformed derivatives contribute to mammary cancers. Furthermore, our system should be useful for lineage tracking, cell-specific gene knockdowns, and other strategies to functionally evaluate putative MSCs and stem cells in other organs thereby providing insight into similarities and differences among the tissue and cancer stem cells in diverse systems.

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P56-8: USE OF siRNA LIBRARY SCREENING TO SYSTEMATICALLY IDENTIFY THE MOLECULES INVOLVED IN RADIORESISTANCE IN BREAST CANCER

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Radiotherapy is routinely applied clinically on patients with localized high-risk breast cancer to enhance the therapeutic outcome and patient survival. A major reason for radiotherapy failure is an incomplete elimination of the residual cancer cells. Recent studies now clearly identify cancer stem cells as the source of this resistance due to the nature of their differential responses to irradiation. This project will systematically investigate the molecules that are involved in radioresistance and are critical for sensitization to radiation treatment in breast cancer. We are applying a high-throughput screening system (siRNA library screening) using the unique breast cancer stem cells to achieve these goals. We have identified a core at M. D. Anderson which will perform the screening for using a genome-wide siRNA library. Currently, we are testing that liposome has the best transfection efficiency for using robotic system to perform reverse transfection in our cells. We are also doing the assay development for this large-scale screening. After we identify the candidate siRNA oligos that will sensitize the radioresistance in breast cancer stem cells, we will confirm the results using other breast cancer cell lines. Thus, the success of our studies will provide the first step toward the development of novel therapeutic strategies for elimination of radioresistant breast cancer cells and as a result, lead to significant reduction of breast cancer death.

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P56-9: CIRCULATING TUMOR CELLS AND STEM CELLS IN BREAST CANCER

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Background and Objectives: The detection and quantification of cancer cells within the blood of patients with metastatic breast cancer correlates with survival, suggesting that some circulating tumor cells (CTC) may functionally act as cancer stem cells (CSC). Current methods used to identify CTC lack the capacity to isolate living cells. Thus, the functional relationship between CTC and CSC remains unclear.

Methods: Clonogenic breast CSC were initially defined using the cell surface antigens CD44 and CD24. More recently, relative expression of the intracellular enzyme retinaldehyde dehydrogenase (ALDH) has been found to distinguish breast CSC from their more differentiated progeny similar to other normal and malignant tissues. We hypothesized that the combination of surface antigens and ALDH activity is capable of identifying and isolating CSC from the peripheral blood of patients with advanced breast cancer. We have begun these studies and stained peripheral blood mononuclear cells isolated by density centrifugation with the Aldefluor reagent as well as monoclonal antibodies against CD24, CD44, and CD45 followed by flow cytometric analysis. We also used the specific enzyme inhibitor diethylamino-benzaldehyde to define cells that exhibit ALDH activity.

Results to Date: We have developed a sequential gating strategy to detect circulating cells with the putative CSC phenotype from patients with advanced breast cancer. Cells that lack the expression of CD45 (i.e., blood cells) and CD24 (since breast CSC do not express this antigen) are initially gated (Figures A and B). This population is then subsequently analyzed for the CD44 expression and Aldefluor (Figures C and D). We have been able to detect CD44⁺CD24^{neg}ALDH^{high} cells in the circulation of breast cancer patients (box, Figure D) whereas these cells are not present in the blood of normal controls (Figure C).

Conclusions: CTC from breast cancer patients appear to be detectable using combinations of surface antigen expression and ALDH activity. Studies are ongoing to verify the epithelial origin of these cells and examine their growth properties in vitro. These results may establish a relationship between CTC and CSC as well as serve as a novel means of utilizing CSC as a novel biomarker in breast cancer.

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P56-10: Met SIGNALING IN MAMMARY STEM CELL PROLIFERATION

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An increasing number of studies suggest that mammary stem cells (MaSCs) are the cellular origin of breast cancer and a source of therapeutic resistance. A clearer understanding of MaSC proliferation and differentiation is essential for successful breast cancer treatment. Because the receptor tyrosine kinase Met is involved in mammary gland development and breast cancer progression, Met is a promising candidate as a regulatory gene of MaSC proliferation. To determine whether activated Met increases stem cell proliferation, we are isolating and measuring MaSC populations in developing mammary glands and mammary tumors from both wild-type (WT) mice and novel mouse models of mutationally activated Met (Met^{mut}). The Met^{mut} mouse models contain activating mutations within the tyrosine kinase domain of Met (M1248T, Y1228C, and M1248T/L1193V). We are currently isolating MaSCs from Met^{mut} and WT mice using fluorescence-activated cell sorting (FACS) of CD45⁺CD31⁺TER119⁺CD29⁺CD24⁺ cells. Isolated MaSCs are then transplanted into cleared fat pads of 3-week-old mice, and epithelial outgrowths are measured to determine stem cell activity. Transplantation experiments are under way for M1248T, Y1228C, and M1248T/L1193V Met^{mut} lines. Since isolated MaSCs are composed of heterogeneous cell populations, we will further determine if Met has a cell autonomous effect on MaSC proliferation by enriching for Met⁺ and Met⁻ MaSCs. In the near future, Met⁺ and Met⁻ cells will be enriched from MaSCs via FACS, and Met expression will be confirmed using an immunofluorescence staining with a Met-specific antibody. Stem cell activity will be measured in Met⁺ and Met⁻ MaSCs by colony-forming assays and mammary fat pad transplantation assays.

Met is a key therapeutic target for numerous cancers including breast cancer. Understanding the temporal and cellular origin of Met activity in breast cancer is critical for developing successful therapies. The proposed studies will use an in vivo model to determine whether Met is involved in the regulation of mammary stem cells. If our hypothesis is confirmed, this would further our understanding of the connection between stem cells and cancer. Moreover, a positive role for Met in MaSC proliferation would indicate that treatment with Met inhibitors during the earliest stages of breast cancer is vital for prevention of tumor progression and resistance to treatment.

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P56-11: STEM CELLS THAT GIVE RISE TO BREAST CANCER MAY HAVE ORIGINATED IN THE BONE MARROW

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In both inherited and acquired human breast cancer, actual cancer development is rare. Even when all breast cells contain inherited BRCA mutations or are exposed to estrogen/radiation, breast cancer develops from only a single transformed cell. The breast does however contain multifocal ductal proliferations. These findings have been interpreted by hypothesizing that the multifocal proliferations reflect a first "hit" but the rare cancer emerges from additional "epithelial hits." But this hypothesis may not be correct. Emerging evidence that transformed stem cells may be the source of human cancers may explain the rareness of breast cancer. We recently studied 14 patients who had received a different sex donor marrow transplant for lymphoma/leukemia who later developed a solid cancer. In 7 patients (2 breast cancers), based on X/Y chromosomal FISH and LOH studies, the solid cancer was of donor origin. These results suggested that the donor's marrow might contain the cell of breast cancer origin. We felt that this novel observation/hypothesis needed to be tested experimentally. We felt that genetically engineered breast cancer mouse models were ideally suited to do this and proposed to conduct murine bone marrow transplant experiments. For our first objective, we introduced "genetically marked" bone marrow derived from tumor-prone mice into either normal healthy or unmarked tumor-prone mice. For our second objective, we are in the process of introducing bone marrow from "genetically marked" healthy mice into unmarked tumor-prone mice. In all the groups we monitored tumor onset and genetically assessed the origin of the cancers that arose: donor or recipient. For our first objective, we used two different murine female bitransgenic breast cancer models: (1) the highly penetrant very robust though somewhat unnatural *MMTV-pyMT*/Rosa26 where polyomavirus middle T was overexpressed as well as the lacZ reporter gene and (2) the less penetrant but more natural *MMTV-erbB2/neu*/Rosa26. Marrow from each of these mice was transplanted into sublethally irradiated normal mice as well as their respective unmarked transgenic recipients. In the former group emerging breast cancers of donor origin were observed in 10% of the mice. In the latter recipients, breast cancers of donor origin were observed in 20% of the mice occurring adjacent to multifocal ductal hyperplasias as well as some cancers of recipient (unmarked) origin. We are in the process of excluding fusion of donor bone marrow-derived lymphocytes with mammary epithelium as the explanation for our findings. We have not yet fully answered our second question: whether introducing bone marrow from "genetically marked" healthy mice into unmarked tumor-prone mice reduces breast cancer development. If our initial findings continue to be borne out, it will indicate that the traditional "multihit epithelial" hypothesis of breast cancer origin may be incorrect. Rather our findings will indicate that clonal breast cancers may arise from rare stem cells that initially originated in the bone marrow. If our hypothesis that the bone marrow contains breast cancer stem cells can be confirmed, we plan to use cell-type specific marker analysis and genetic approaches to identify the putative marrow-derived stem cells that give rise to breast cancer.

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P56-12: ROLE OF BONE MARROW-DERIVED CELLS IN BREAST CANCER

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Background and Objectives: Experimental and clinical evidence indicates that acquisition by cancer cells of epigenetic and phenotypic features of normal stem cells (NSC) is a hallmark of tumor stem cells, which are essential for sustained tumor progression, metastasis, and manifestation of clinically lethal therapy-resistant malignant phenotypes. Recent observations demonstrate that bone marrow-derived cells (BMDC) can contribute to epithelial cancers. To test the hypothesis that BMDC play a role in breast cancer progression and metastasis, we employed the HER-2/neu transgenic mouse model of mammary carcinogenesis.

Methodologies: HER-2/neu mice (recipients) were lethally irradiated and reconstituted with BM from male donor mice genetically marked with green fluorescent protein (GFP). Engraftment of BMDC in mammary gland tumors was monitored by anti-GFP staining. Mammary tumors and selected normal tissues from sacrificed mice were processed for recovery of viable cell cultures as well as histologic, immunohistochemical, and immunofluorescence analyses.

Results: Lethal irradiation and bone marrow transplantation protocols did not alter the incidence and growth of breast tumors. Our analysis demonstrates the consistent presence of BMDC in 100% of examined breast tumors and seems to indicate the possible involvement of BMDC information of stromal components and blood vessels in breast tumors. Stromal elements recovered from transgenic breast tumors enhance malignant potential of tumor-initiating epithelial breast cancer cells highlighting novel malign-

nancy-promoting cooperating mechanisms between the progenies of mesenchymal and epithelial stem cells.

Conclusions: Results of our experiments do not support the hypothesis that BMDC make direct contribution to the malignant epithelial components of breast tumors. Our experiments provide important methodological frameworks for systematic experimental analysis of the role of BMDC in breast cancer and set up the stage for a new area of investigation in basic and clinical breast cancer research.

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P56-13: CHARACTERIZATION OF THE RADIORESISTANCE OF STEM-CELL LIKE CELLS IN BREAST CANCER CELL LINES

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The presence of cancer stem cells may explain cancer initiation and recurrence after treatment. To demonstrate stem cell-like cell (SCLC) subpopulations in breast cancer cell lines, we examined by flow cytometry Hoechst 33342 efflux, ABCG2 protein, and CD44/CD24 surface markers. Hoechst 33342 efflux identified stem cells in MCF-7 (1.3%), MDA-MB-436 (0.6%), MDA-MB-231 (0.4%), and HCC1937 (0.1%) but none in MDA-MB-453 and HCC38 cells. The presence of ABCG2 correlated with Hoechst 33342 efflux in MCF-7 and HCC1937 but not MDA-MB-231 cells, suggesting that ABCG2 activity responsible for Hoechst 33342 efflux may be cell line specific. Analysis of CD44 and CD24 expression, known markers of SCLC, showed a subpopulation of CD44⁺/CD24⁺ cells ranging from 97% and 55.3% positive cells in mesenchymal-like breast cancer cell lines MDA-MB-436 and MDA-MB-231 to 0% to 5.9 % in epithelium-like cell lines, MCF-7 and HCC1937. To determine if the CD44⁺/CD24⁺ population varies with time, the CD44/CD24 surface markers were assayed at 6 days and 2 weeks of culture after sorting. The CD44⁺/CD24^{or low} (MCF-7, MDA-MB-231, and HCC1937 cells), CD44⁺/CD24⁺ (MDA-MB-231), and CD44⁺/CD24^{high} (MCF-7 and HCC1937) populations were reanalyzed after 6 days of culture with 70%–80% of cells maintaining the original CD44/CD24 phenotype. After 2 weeks of culture the following transitions occurred: (1) CD44⁺/CD24^{or low} cells changed to CD44⁺/CD24^{high} cells (MCF-7 and HCC1937) or to CD44⁺/CD24⁺ (MDA-MB-231) and (2) CD44⁺/CD24^{high} (MCF-7 and HCC1937) and CD44⁺/CD24⁺ (MDA-MB-231) cells became CD44⁺/CD24^{or low} cells. As SCLC are radiation resistant, we examined the radiation sensitivity of CD44⁺/CD24^{or low} cells with a clonogenic assay on sorted populations of MCF-7, MDA-MB-231, and HCC1937 cells. Increased radiation resistance was seen in the CD44⁺/CD24^{or low} subpopulations compared to CD44⁺/CD24^{high} (MCF-7 and HCC1937), CD44⁺/CD24⁺ (MDA-MB-231) cells, and nonsorted cells. Analysis of the survival fraction at 2Gy showed the survival fraction in CD44⁺/CD24^{or low} subpopulation of all three cell lines increased by >40% compared to the CD44⁺/CD24^{high} (MCF-7 and HCC1937) and CD44⁺/CD24⁺ (MDA-MB-231) cells. Increased radiation resistance was not a function of nonhomologous end joining (NHEJ) as there was no in vivo change in NHEJ activity in CD44⁺/CD24^{or low} cells nor altered expression of the NHEJ-related proteins Ku 80, Ku70, PARP-1, and DNA-PK. Differences were found in the activity of ATM pathway between CD44⁺/CD24^{or low} and the CD44⁺/CD24^{high} (MCF-7 and HCC1937) or CD44⁺/CD24⁺ (MDA-MB-231) subpopulations, suggesting that radiation resistance of SCLC might result from altered ATM activity. These studies give credence to the hypothesis for SCLC in breast cancer cell lines that properties of these populations vary between cell lines and that radiation resistance of SCLC may be from altered ATM pathway.

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P56-14: IDENTIFICATION AND CHARACTERIZATION OF CXCR4-POSITIVE BREAST CANCER STEM CELLS

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Background: Recently, breast tumor-initiating cells/stem cells expressing either cell surface markers (CD44⁺/CD24^{low}) or epithelial-specific antigen (ESA+) but not sialomucin (MUC-) were isolated from breast tumors. The chemokine receptor CXCR4 has been shown to be expressed on hematopoietic stem cells as well as on non-hematopoietic tissue committed stem cells (TCSCs). Furthermore, CXCR4 and its ligand CXCL12 have been found to play an important role in the survival as well as trafficking of various types of TCSCs. In addition, the CXCR4/CXCL12 axis has been shown to regulate breast cancer metastasis.

Objective: The purpose of this study is to identify CXCR4-positive progenitor/stem cells and to characterize their migratory and chemoinvasive properties.

Methodology and Results: We have analyzed various breast cancer cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-468, MCF-7, and DU4475) for the expression of stem/progenitor cell antigens such as CD44, CD24, ESA, and Muc. Next we analyzed CXCR4 and pro-invasive gene expression in these cell lines. Only two cell lines, MDA-231 and MDA-435, showed stem/progenitor cell phenotype expression (CD44⁺/

CD24^{low}). Although DU4475 cells had high amounts of ESA, they also possessed a significant amount of Muc antigen. Out of these cell lines, the MDA-231, MDA-435, and DU4475 cells exhibited high chemotaxis in the presence of serum whereas the MDA-231 and MDA-435 cells were highly invasive as shown by matrigel-coated basement membrane assays. Studies involving CXCR4 expression revealed that 40% to 60% of the MDA-231 and DU4475 cells expressed CXCR4. We did not find any correlation between stem/progenitor cell phenotype and CXCR4 expression as the MDA-435 cells were also negative for CXCR4 expression as shown by FACS analysis. Analysis of genes involved in invasion revealed that cells showing the stem/progenitor phenotype also expressed high amounts of uPAR whereas other cells had low uPAR expression. Next we isolated two different populations of cells, CD44⁺/CXCR4- and CD44⁺/CD24- from tumors and analyzed their metastatic properties in nude mice. Mice injected with either of the populations (CD44⁺/CXCR4- and CD44⁺/CD24-) individually showed no metastasis. However, mixed population of these cells showed lung metastasis. In addition, we have also analyzed tumor-suppressive properties of Slit-2, which we have shown to block CXCR4/CXCL12-mediated functional effects. Slit-2 overexpressing MCF-7 cells exhibited decreased proliferation and migration properties in vitro and, when injected in mice, demonstrated a 60% to 70% reduction in tumor size as compared to vector control MCF-7 cells.

Conclusions: In summary, human breast cancer cells with a stem cell phenotypic marker show a higher chemotactic/invasive potential in the presence of serum. These cell lines also express high amounts of the pro-invasive gene, uPAR. However, no direct relationship was observed between the stem/progenitor cell phenotype and CXCR4 expression. In addition, we have shown that a novel molecule, Slit-2, which modulates CXCR4 function, inhibits breast cancer tumor growth in vivo. Our studies provide new information regarding correlation between CXCR4 expression and stem/progenitor cell phenotype and tumor suppressive activity of Slit-2 in breast cancer.

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P56-15: DETECTING BREAST CANCER (BRCA) STEM CELLS AMONG CIRCULATING CYTOKERATIN (CK)+ HEA+ CELLS

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Background and Objectives: Detection of HEA+ CK+ cells in the circulation has been shown to correlate with poor prognosis in stage 4 BrCA. Circulating HEA+ CK+ cells may represent mature (nontumorigenic) cells shed from occult tumor, or alternatively, tumorigenic cells that may mediate metastasis. To distinguish between these alternatives, we have developed an approach incorporating immunomagnetic separation and multi-parameter flow cytometry in an attempt to detect the BrCA tumor stem cell phenotype (CD90+ CD44+ CK+) among HEA+ CK+ cells in the peripheral circulation.

Methodologies: Cryopreserved clinical material (3 normal and 3 BrCA mobilized peripheral blood progenitor cell leukapheresis products) were separated for HEA+ (a.k.a. EpCAM+) cells on an AutoMACS immunomagnetic device. As a positive control, 3 BrCA bone marrows (BM) were also separated. An average of 5.2 x 10⁸ cells were separated. HEA+ and unseparated cells were counted and stained for flow cytometric analysis of stem and differentiation markers (nucleated cells: DAPI; Hematopoietic: CD45-APC.Cy7, CD14+CD33+glycophorin-PE.Cy5; Epithelial: HEA-APC, intracellular pancytokeratin (CK)-FITC; Stem/Progenitor: CD90-PE.TxRed, CD117-PE.Cy7, CD44-PE). HEA separated cells were acquired exhaustively, and 2–10 million unseparated cells were acquired on a CyAn cytometer. Spectral compensation and analysis were performed offline using a prototype version of VenturiOne software. Circulating BrCA cells were identified as having at least 2N DNA (DAPI), CD45/CD33/CD14/glycophorin negative, HEA+ and CK+, a profile similar to that used by an FDA approved commercial assay (CellSearch).

Results to Date: Average purity of HEA separated cells was 32%, a 222-fold enrichment. The lower limit of detection of HEA+ CK+ cells, estimated as the 99th percentile of normal pheresis, was 0.02%. Two of 3 BrCA leukapheresis products had significant HEA+ CK+ populations by this criterion (0.70% and 0.23%). Two of 3 separated BrCA BM were also positive for HEA+ CK+ cells (0.28% and 0.33%). Analysis of stem/progenitor markers on HEA+ CK+ cells in the 2 positive leukapheresis products revealed 0.5–2.3% CD90+ and 0.5–2.6% CD133+. These populations were largely nonoverlapping.

Conclusions: We have used cryopreserved discarded clinical materials to develop a method to evaluate the expression of stem/progenitor cell markers on rare circulating HEA+ CK+ cells. In this small sample, circulating HEA+ CK+ cells were detected, a fraction of which bore immunophenotypic markers consistent with stem/progenitor cells. This assay can be applied to leukapheresis, large-draw peripheral blood, and BM samples to determine whether the presence of circulating (and presumably clonogenic) BrCA stem/progenitor cells is indicative of disease progression.

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CELL CYCLE

Poster Session P57

P57-1: CKS1 SENSITIZES BREAST CANCER CELLS TO CHEMOTHERAPEUTIC AGENTS

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Background and Objectives: A major challenge in the fight against breast cancer remains the ability to treat tumors that can escape currently used therapeutic regimens (i.e., drug resistance). Breast cancer often occurs when the proteins that regulate normal epithelial cell division become dysregulated. In this study, we examined the role of the cell cycle regulatory protein, called human cyclin-dependent kinase subunit 1 (Cks1) in human breast cancer. Specifically, we are investigating whether Cks1 expression alters the growth of breast cancer cells and/or sensitizes breast cancer cells to the effects of chemotherapeutic agents.

Methodologies: MCF-7 breast tumor cell lines were created that stably overexpress Cks1 or Cks2 at various levels. The Cks1 or Cks2 overexpressing MCF-7 cells were then treated with chemotherapeutic agents (5-fluorouracil, methotrexate, and taxol). The growth inhibitory and apoptotic effects of Cks1 were then evaluated in vitro and in vivo. Furthermore, the ability of Cks1 to overcome resistance to chemotherapeutic agents was examined by either overexpressing Cks1 in breast tumor-derived cell lines that were either resistant to 5-fluorouracil or methotrexate, two commonly used chemotherapeutic agents for breast cancer treatment.

Results: We have shown that Cks1 expression results in growth suppression of breast cancer cells and suppressed xenograft tumor growth in vivo through induction of apoptosis. Overexpression of Cks1 significantly sensitizes breast cancer cells to chemotherapeutic agents through the induction of apoptosis. Furthermore, Cks1 overexpression was shown to overcome the resistance to chemotherapeutic agents in these breast cancer cell lines. In support of our in vitro and in vivo studies, pathologic analysis of human breast tumor samples revealed that an elevated Cks1 protein level is significantly associated with tumor differentiation and a positive response to adjuvant chemotherapy.

Conclusions: This study is the first to report that an elevated level of Cks1 in breast cancer cells sensitizes them to chemotherapeutic agents. Our results indicate that Cks1 is an important mediator of the therapeutic response of breast cancer cells that could potentially be exploited as a broad spectrum molecular target to sensitize breast cancer cells to the effects of chemotherapeutic agents.

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P57-2: CDK INHIBITOR p18INK4c CONTROLS MAMMARY LUMINAL PROGENITOR CELL PROLIFERATION AND TUMORIGENESIS

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Breast cancer is a heterogeneous disease comprised of several histopathologically and molecularly distinct tumor types diverse in their responsiveness to treatment. We report here that deletion of the cyclin-dependent kinase (CDK) inhibitor p18INK4c, a haploinsufficient tumor suppressor whose expression is inactivated or reduced in several types of human cancers, stimulates luminal cell proliferation in mammary glands and results in the development of ER-positive luminal tumors at a near complete penetrance. p18 deficiency causes an expansion of mammary stem cells at a young age in mice but premature depletion in adult. In contrast, luminal progenitor cells are expanded in young mice and maintained throughout adulthood by the p18 loss. In human breast cancers, low p18 expression is associated with luminal A tumors. These results demonstrate p18INK4c controls mammary luminal progenitor cell proliferation and tumorigenesis.

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P57-3: INVESTIGATING CYCLIN G2 EXPRESSION, LOCALIZATION, AND CELL CYCLE EFFECTS IN BREAST CANCER CELLS

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Estrogenic signals play an important and central role in the promotion and development of the most frequent type of premenopausal breast cancer (BC), sporadic BC. Because aberrant pro-growth signals from estrogen hormones and growth factor receptors play an important role in the promotion and development of breast cancer, the hormone and growth factor receptors that transmit these signals are key molecular targets of the major therapies used to treat breast cancer. Tamoxifen and trastuzumab (Herceptin) are the two best known of these therapeutics. Despite their usefulness, breast cancers eventually develop resistance to them. Thus improved understanding of how they act to diminish breast cancer growth may allow for new more effective treatment strategies to

be developed. Importantly, expression of the unconventional, FOXO-regulated growth inhibitory cyclin, that is the focus of this study, cyclin G2 (G2), is repressed downstream of these receptor signaling pathways. Indeed, basal transcription of CCNG2, the gene encoding G2 is directly repressed by ligand-bound ER. Accordingly, G2 expression levels may indicate the effectiveness of ER antagonistic therapies.

Chromosomal instability (CIN) and aneuploidy are prominent characteristics of early in situ carcinoma and primary invasive ductal BCs. Centrosome amplification strongly correlates with CIN and is thought to indicate genetic instability and tumor aggressiveness. Notably, recent studies link E2 treatment of mice to centrosome amplification, CIN, and aneuploidy. Importantly, we determined by microscopy and biochemical analysis that exogenous and endogenous G2 are primarily localized to the centrosome in normal and transformed cells. Our novel observation that basal G2 is a centrosomal protein suggests that upon elevation it exerts its inhibitory effects through a centrosomally linked process. Repression of G2 expression by E2-induced signals may thus inhibit G2-modulated centrosomally linked cell cycle control. We hypothesized that (1) G2 is normally upregulated during stress-responses of breast epithelial cells to promote cell cycle exit; (2) high E2 represses G2 upregulation, which in turn, further promotes BC cell proliferation. The results from the studies supported by this grant provide evidence that (1) ectopically expressed G2 is growth inhibitory for BC cell lines, (2) basal G2 protein levels in cells derived from normal breast epithelia and BC are tightly regulated through estrogenic signaling pathways, (3) estradiol treatment of MCF-7 cells represses G2 expression, and its localization to centrosomes and the nucleus, whereas growth inhibitory antiestrogenic signaling via selective estrogen receptor modulators (SERMs), upregulates G2 protein expression, and accumulation at both subcellular sites. Moreover our recent biochemical and cell biological studies suggests that cyclin G2 expression is not only upregulated in breast cancer cells by therapeutics that inhibit estrogenic signals, but also those that target growth factor-induced proliferation signals, and traditional chemotherapeutics that target tumor DNA. These results provide the framework for further studies to investigate cyclin G2 function in growth control of breast epithelia and whether cyclin G2 could be an important biomarker for disease prognosis and treatment efficacy in breast cancers.

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P57-4: CLOCK AND CLOCK-CONTROLLED GENE EXPRESSION IN MOUSE MAMMARY TISSUE

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Epidemiological studies have linked shiftwork with an increased risk of breast cancer, suggesting that circadian rhythm disruption may influence the development or progression of the disease. Circadian rhythms are regulated by a panel of specific transcription factors, called clock genes, and our current understanding of endogenous cellular rhythmicity is that both positive and negative feedback cycles of clock genes drive the expression of a growing list of other transcription factors and functional genes. Disruption of circadian control systems, which maintain normal cell function, may lead to malignant transformation into cancer cells. This concept is supported by recent findings that tumors grow faster in arrhythmic mice. Previous studies have demonstrated rhythmicity of expression of clock genes in mouse liver, heart, kidney, and vascular tissue. As the patterns of expression of genes induced by these cyclic transcription factors were highly tissue specific, it is critical to determine which clock and clock-controlled genes are expressed in mammary tissue and potentially contribute to the increased risk of breast cancer associated with shift work.

General hypothesis: Circadian disruption of clock gene rhythmicity in mammary tissue will result in altered gene expression and lead to the loss of growth inhibition and subsequent tumorigenesis.

Experimental aim: To measure the expression of clock and clock-controlled genes in mammary tissue.

Real-time RT-PCR was used to investigate the mRNA expression of selected genes in mammary tissue of 6-week-old virgin Balb/c female mice every 3h for 24h.

We measured the expression of clock genes and *c-myc*, *wee1*, *Gadd45a*, *Cyclin D1*, *tgfb1*, *erb b2*, *vegfr*, *bcral*, and *timp* in mouse mammary tissue. *Bmal1*, *Clock*, *per1*, *per2*, *cry1*, and *cry2* mRNA changed 2.7–9.1-fold across 24 hours ($P < 0.05$) with peak expression of *Bmal1* and *Clock* occurring during darkness and *per1* and *per2* occurring 12 hours later. *c-myc*, *wee1*, *Gadd45a*, *Cyclin D1*, *tgfb1*, and *erb b2* mRNA also changed significantly 2.1–3.1 fold, peaking in darkness, while expression of *vegfr*, *bcral*, and *timp* mRNA did not change. Genes involved in mammary development, function, and tumorigenesis are not constitutively expressed across the day, and disruption of this pattern may contribute to the development of cancer in shift workers.

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P57-5: ABSTRACT WITHDRAWN

P57-6: DISRUPTION OF THE CIRCADIAN RHYTHMS OF GENE EXPRESSION AND THE DEVELOPMENT OF BREAST CANCER

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A recent systematic review of the literature has highlighted a link between long-term shiftwork and an increased risk of breast cancer incidence. The mechanisms underlying this association are unclear and consequently, the focus of the current project is to gain a better understanding of the role that the circadian time-keeping system might play in the development and/or progression of breast cancer. Our general hypothesis is that shiftwork disrupts endogenous circadian rhythms and this facilitates oncogenesis.

Many cellular and physiological processes are temporally regulated by the circadian time-keeping system. Every cell expresses a suite of at least 8 genes, known as clock gene transcription factors whose protein products participate in negative and positive feedback loops that unfold over a 24-hour period. These feedback loops engage with other transcription factors and appear to control at least 10% of the transcriptome according to some recent microarray studies. As an example, genes that play a pivotal role in the genesis and progression of breast cancer, such as *cMyc* or *Wee1* are under the control of the circadian time-keeping system and consequently offer a putative pathway to explain some of the negative consequences of shiftwork on the incidence of breast cancer observed in female shiftworkers.

The objectives of the current project are to determine:

1. How simulated shiftwork affects the expression of clock genes and genes involved in the control of the cell cycle in murine mammary tissue.
2. The effect of rhythm disruption on the growth and gene expression of MCF-7 xenografts in immunocompromised mice and tumor development in PyMT mice.
3. The phase relationship of the circadian time-keeping system between host and MCF-7 xenografts.
4. How impairment of clock gene expression in human breast cancer cell lines affects cell division and apoptosis.

Methodologically the project uses a shiftwork simulation protocol whereby mice are subjected to a 12-hour reversal of their photoperiod every 3 days over 1 month. On day 28, one night after the latest photoperiod reversal, the mice are sacrificed every 4 hours over a 24-hour period and mammary tissue and/or xenografts are collected for analysis of gene expression by real-time RT-PCR and protein levels by western blotting.

Shiftwork simulation impacts significantly on the expression of core clock genes and the cell-cycle gene *Wee1* in murine mammary tissue. We have also shown that MCF-7 xenografts express clock genes rhythmically in immunocompromised mice. Experiments are under way to determine if the xenografts maintain rhythmicity in mice with a *Clock* mutation rendering the host arrhythmic. Finally, we have impaired clock gene expression by knocking down the expression of *Bmal1* in MCF-7 and MCF-10A cell lines and shown a small but significant effect on cell proliferation and apoptosis.

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P57-7: TOWARD DESIGN OF A BISUBSTRATE ANALOG INHIBITOR OF CYCLIN A-cdk2

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Cell division is driven by a series of Ser/Thr kinases known as cyclin-dependent kinases. An important question in the cell cycle field is how cdk2 recognize their substrates, and an important question in cancer research is how to inhibit these interactions. The overall objective was to characterize a novel Cyclin A-cdk2-substrate interaction and to use this information to design an inhibitor that blocks multiple substrate interaction sites on Cyclin A-cdk2. We obtained definitive data for a novel interaction between Cyclin A-cdk2 and this substrate—peptides derived from Cyclin A interacting proteins such as the substrate E2F1 or the inhibitory protein p21, which inhibit phosphorylation of many Cyclin A-cdk2 substrates, do not inhibit phosphorylation of this substrate. This year, we found that another substrate, p107, can coexist in a complex with Cyclin A-cdk2 and this substrate, underscoring the importance of characterizing this novel interaction. Over the course of the award, much progress has been made at understanding Cyclin A-cdk2-mediated phosphorylation of this substrate, and we have obtained data in cells that underscore importance for cell division.

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P57-8: PHOSPHORYLATION OF CENTRIN BY AURORA A DRIVES CENTROSOME AMPLIFICATION IN CANCER

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Aurora A is an oncogenic serine/threonine kinase which when over-expressed can cause cell transformation and centrosome amplification. Centrin is a small calcium-binding phospho-protein, which is essential for centrosome duplication. Here, we show that aurora A phosphorylates centrin at serine residue 170 and a recombinant centrin mutation that mimics this phosphorylation results in centrosome amplification in part by increasing centrin's stability against APC/C-mediated proteasomal degradation. Analysis of human breast cancers reveals high levels of aurora A expression in invasive tumors and the accumulation of phospho-centrin at supernumerary tumor centrosomes. Additionally, over-expression of aurora A in cells expressing only nonphosphorylatable centrin abrogates the ability of aurora A to cause centrosome amplification. Taken together, these results indicate that aurora A over-expression leads to phosphorylation and stabilization of centrin, which in turn drives to centrosome amplification in cancer.

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P57-9: pRB FAMILY PROTEINS AND THE EPIGENETIC CONTROL OF ESTROGEN RECEPTOR- α : A NOVEL AND INTRIGUING MECHANISM IN BREAST CANCER

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Estrogen receptor- α (ER- α) plays a crucial role in normal breast development and has also been linked to mammary carcinogenesis and clinical outcome in breast cancer patients. However, ER- α gene expression can change during the course of disease and consequently therapy resistance can occur. The molecular mechanism governing ER- α transcriptional activity and/or silencing by chromatin remodeling in breast cancer cells is still unclear.

However, our studies show that the presence of specific pRb2/p130-macromolecular complexes strongly correlate with the methylation status of ER- α gene. Furthermore, we suggest that pRb2/p130 could cooperate with ICBP90 (Inverted CCAAT box Binding Protein of 90 kDa) and DNMTs (DNA methyl transferase) in maintaining a specific methylation pattern of ER- α gene in breast cancer cells.

Our novel and intriguing hypothesis is that the sequence of epigenetic events for establishing and maintaining the silenced state of ER- α gene can be locus or pathway specific, and that the remodeling of local chromatin structure of ER- α gene by pRb2/p130-multimolecular complexes may influence its susceptibility to specific DNA methylation. Our data could provide a basis for understanding how the complex pattern of ER- α methylation and transcriptional silencing are generated, and for understanding the relationship between this pattern and its function during the neoplastic process. Moreover, our results may provide a key to understanding the mechanism regulating ER- α expression during the neoplastic events and then provide new targets for designing novel therapeutic strategies.

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P57-10: Cdk2 PHOSPHORYLATION ON THREONINE 39 BY PKB (AKT) AND ITS IMPLICATION ON CYCLIN BINDING, CELLULAR LOCALIZATION, AND CELL CYCLE PROGRESSION

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Cdk2 importantly regulates G₁ progression. Cdk2 activation requires cyclin binding and this facilitates the CAK-mediated Cdk2 phosphorylation at T160 needed for full Cdk2 activity. Here we describe a novel Cdk2 site whose PKB-dependent phosphorylation appears to facilitate cyclin-Cdk2 assembly. PI3K inhibition caused cyclin E and A disassociation from Cdk2, loss of CAK phosphorylated Cdk2, and G₁ arrest. Cdk2 bears a PKB consensus motif containing threonine 39 (T39) immediately preceding the PSTAIRE helix. Cellular Cdk2 co-precipitated with PKB and PKB phosphorylated Cdk2 in vitro. Transfected Cdk2T39A showed reduced association with cellular cyclin whereas phosphomimetic Cdk2T39E showed an increased association with cellular cyclin. Cdk2 phosphorylation by PKB increased its ability to bind cyclins E and A in vitro. Based on these data and the previously solved Cdk2-cyclin structures, we propose a model whereby T39 phosphorylation of Cdk2 would induce a conformational change to facilitate cyclin binding and subsequent CAK action.

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P57-11: RRP1B IS A SPECIFIC E2F1 TRANSCRIPTIONAL TARGET

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E2F1 belongs to the E2F family of transcription factors important for control of cellular proliferation. Dysregulation of E2F is an event in most, if not all cancers. Interestingly, while E2F1 plays an important role in proliferation, E2F1 can also induce apoptosis, an apparently paradoxical effect. Induction of specific E2F1 targets in response to specific stimuli thus may determine whether E2F1 promotes proliferation or induces apoptosis. Our laboratory is interested in the molecular mechanisms by which a cell responds to DNA damage. E2F1 plays an important role in the response to genotoxic stimuli; DNA damage activates and stabilizes E2F1 and induces transcription of proapoptotic E2F responsive targets, such as p14ARF, p73, Apaf-1, caspase-3, and caspase-7.

To further study the role of E2F1 in control of apoptosis, our laboratory screened for genes that are specifically induced by E2F1 from previously published microarray data. We identified RRP1B as a novel and specific transcriptional target of E2F1. High expression of RRP1B has recently been shown in *in vitro* studies to reduce the size of both primary tumors and lung metastasis of breast cancer cell lines. RRP1B expression is specifically upregulated by E2F1 overexpression but not other E2F family members. RRP1B expression is correlated with E2F1 expression during the cell cycle. The minimal RRP1B promoter region responsive to E2F1 was identified. Finally, E2F1, but not other E2F family members, was shown to bind endogenous RRP1B promoters through chromatin immunoprecipitation assays. Together, this data suggests that RRP1B is an E2F1 specific target that can regulate tumor progression. Future studies will determine the effect and mechanism of RRP1B in regulation of E2F1-induced apoptosis.

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P57-12: CELL CYCLE REGULATORY ROLES OF ER α IN BREAST CANCER

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Breast cancer is one of the leading causes of death in women worldwide and approximately one in nine women will develop this disease during their lifetime. Clinical, epidemiological, and biological evidence indicate that estrogens participate in the initiation and progression of breast cancer. The mitogenic effects of estrogens on breast epithelial cells are mediated, at least in part, via the estrogen receptor (ER). The roles of estrogen and ER in normal mammary gland development and in transformation to a tumor phenotype have been extensively studied. However, although the cumulative data suggests an important role for ER- α (ER α) in breast cancer, the function of ER α in regulating cell proliferation in normal and tumor tissue remains unclear. This study investigates the role of ER α on the progression of the cells through different phases of the cell cycle in the presence and absence of its ligand, estradiol (E2). The cellular localization of ER α is also examined during cell cycle transition in MCF-7 cells. The major objective of the proposed research is to investigate the role of ER α on the progression of the cells through different phases of the cell cycle in the presence and absence of the ligand, estradiol. We hypothesize that in the absence of ligand, ER α functions as an inhibitor of cell proliferation and modulates the progression of cells through the cell cycle at G2 and M phases. Specific Aims: (1) Examine the mechanism by which liganded ER α leads to deregulation of cell cycle. (2) Determine the mechanism of ER α cellular translocation. (3) Examine the mechanism of ER α turnover in the cell cycle. For the proposed aims, cells will be synchronized by different arresting agents and the expression of ER α and other key cell cycle regulators will be assessed at each phase of the cell cycle in ER α positive cells. In our preliminary studies, we have found that ER α is cell-cycle regulated with peak levels at S and G2/M. Additionally, in MCF-7 cells liganded ER α results in a significant shortening of S and G2/M phase compared to the effect of non-liganded ER α on the cell cycle. We next questioned if the cell cycle localization of ER is different at each phase of the cell cycle. The preliminary data reveals the presence of ER α in the cytoplasm during the G1 and early S phases of the cell cycle and then at the end of S phase and G2/M phases of the cell cycle ER α translocates to the nucleus. The pattern of translocation of ER α could explain the differential cell cycle regulation during G1-S phases from the G2/M phase. While these results are preliminary, they are suggestive that ER can get translocated to the nucleus in a cell-cycle-dependent fashion. We will further pursue this line of experimentation in aim 3. Lastly we will examine the mechanism underlying the degradation of ER α . Since the expression level of ER α in the cell cycle is similar to the changes of cyclin B1, which its degradation is cell cycle dependent and known to be mediated by Anaphase Promoting Complex (APC). To this end we will examine the involvement of APC in ER α degradation. These experiments will help elucidate the mechanism by which cell-cycle expression of ER α regulates the progression of cells through the cell cycle.

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P57-13: FUNCTIONAL ANALYSIS OF MITOTIC PHOSPHORYLATIONS IN BARD1

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The BRCA1 tumor suppressor has been implicated in numerous cellular processes, including DNA repair, cell cycle checkpoint control, and mitotic spindle assembly. *In vivo*, BRCA1 exists in association with BARD1 and the BRCA1/BARD1 heterodimer is thought to mediate many BRCA1 functions, including its tumor suppression activity. These functions may be regulated in part by post-translational modifications of the heterodimer. We previously showed that the phosphorylation state of BARD1 is cell cycle regulated and that BARD1 is hyperphosphorylated at seven distinct sites during mitosis. The goals of this study are to evaluate the role of BARD1 phosphorylation in cell cycle checkpoints and DNA repair pathways that are dependent on BRCA1, such as the ionizing radiation (IR)-induced G2 accumulation checkpoint and homology-directed repair (HDR) of double-strand DNA breaks (DSB). To study the function of BARD1 phosphorylation in the IR-induced G2 accumulation checkpoint, we used BARD1-specific siRNAs to reduce the expression of endogenous BARD1 in 293 cells and then restored expression with siRNA-resistant wild-type or mutant forms of exogenous BARD1. Western blot analysis indicates that two sequential siRNA transfections with either of two distinct siRNAs resulted in >90% knockdown of endogenous BARD1 and a substantial defect in the IR-induced G2 accumulation checkpoint. This result confirms that BARD1, like BRCA1, is required for activation of this cell cycle checkpoint. Moreover, partial rescue of the checkpoint was achieved upon co-transfection of the siRNA-treated cells with expression vectors encoding siRNA-resistant forms of exogenous wild-type BARD1 mRNA. In addition, checkpoint function was also rescued to a comparable degree with expression vectors encoding siRNA-resistant BARD1 mRNA bearing mutations of the seven specific phosphorylation sites. These results indicate that mitotic phosphorylation of BARD1 is not required for its role in activation of the IR-induced G2 accumulation checkpoint defect. We are currently applying this same approach to determine the function of BARD1 phosphorylation in other BRCA1-dependent IR-induced checkpoints, such as the transient G/M checkpoint, the decatenation checkpoint, and the mitotic exit checkpoint. To evaluate the function of BARD1 phosphorylation in homology-directed repair (HDR) of double-strand DNA breaks (DSB), Bard1-null mouse mammary tumor cells bearing an HDR reporter construct (DR-GFP) were transfected with expression vectors encoding either wild-type or mutant forms of human BARD1. Briefly, this reporter contains two distinct nonfunctional copies of the GFP gene: one copy (SceGFP) is disrupted by the recognition site for the rare-cutting endonuclease I-SceI, while the other copy (iGFP) encodes only an internal region of GFP. However, a functional GFP gene can be regenerated when a DSB break triggered by I-SceI cleavage of the SceGFP is repaired by HDR utilizing iGFP as a template, and such events can be quantified by flow cytometry. Using this assay, we previously showed that transfection of these Bard1-null cells with an expression vector encoding human BARD1 induces an approximately 5-fold increase in HDR function. However, a similar increase in HDR function was readily achieved upon transfection with expression vectors encoding phospho-mimicking and phospho-deficient forms of BARD1, indicating that mitotic phosphorylation of BARD1 is not required for HDR of DSBs.

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P57-14: THE ROLE OF Cdk4 IN erbB2, Ras AND Wnt-1-INDUCED BREAST TUMORIGENESIS

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Breast cancer has the second highest incidence of all types of cancers among women. A common feature of most cancers is disruption of cell cycle progression. The cell cycle constitutes four different phases—G1, S, G2, and M with checkpoints at each phase. *Cyclin-dependent kinase 4* (Cdk4), an important regulator of the G1 phase, is found to be amplified in about 16% of human breast cancers. Cdk4 is active only in the presence of D-type cyclins. Cyclin D/Cdk4 complexes phosphorylate the retinoblastoma (Rb) family of tumor suppressor proteins at specific sites leading to inhibition of the Rb family of proteins. The loss of Cdk4 in mice results in insulin-deficient diabetes and sterility. The introduction of the *Cdk4R24C* mutation that prevents the binding of INK4 inhibitors to Cdk4 in mice leads to the development of tumors in multiple organs including breast tissue starting at an age of 8 to 10 months. The presence of this mutation in humans predisposes them to melanoma. These observations indicate the importance of Cdk4 in cancer.

Overexpression of Cyclin D1 in breast tissue of *Cyclin D1* transgenic mice results in mammary adenocarcinomas indicating the oncogenic role of Cyclin D1 in mammary epithelium. The loss of Cyclin D1 renders mice resistant toward breast tumors induced by *ras* and *erbB2* oncogenes but not those induced by *Wnt-1* and *myc*. Thus, the role of Cyclin D1 in breast cancer is well established, but it is not known if Cdk4 plays a similar role. Therefore, we studied the role of Cdk4 in breast cancer induced by *erbB2*, *ras*, and *Wnt-1* oncogenes. Our studies of the mammary glands derived from *erbB2* trans-

genic mice that were nullizygous for Cdk4 indicated impaired ErbB2-induced proliferative disturbances of mammary epithelium. Further, only 14% of these mice developed breast tumors when compared to 97% of their wild-type transgenic counterparts. On the other hand, our studies on *Wnt-1* transgenic mice indicated that *Wnt-1* induces breast tumors even in the absence of Cdk4 with similar frequency when compared to wild-type transgenic counterparts. Our studies with *ras* transgenic mice show that loss of Cdk4 abrogates Ras-induced proliferative disturbances of the mammary epithelium. Further, none of the *ras* transgenic mice that lost the expression of Cdk4 developed breast cancer when compared to 70% of *ras* mice that express Cdk4. Our study of the role of Cdk4R24C mutation in Ras-induced breast tumorigenesis indicated that *ras* transgenic mice that express Cdk4(R24C) develop breast cancer with a significant delay when compared to *ras* transgenic mice that express wild-type Cdk4. Our studies also indicated a significant increase in apoptosis and senescence in the tumors of *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice, which might be partly due to increased DNA damage response as evidenced by the presence of increased levels of phosphorylated form of histone H2AX.

In conclusion, our studies indicate that Cdk4 is essential for ErbB2 and Ras-induced breast tumorigenesis but not for *Wnt-1*-induced breast tumorigenesis. Our studies also indicate that the R24C mutation of Cdk4 does not accelerate Ras-induced breast tumorigenesis. Thus, inhibitors targeting Cdk4 might help to treat breast cancer induced by *erbB2* and *ras* oncogenes.

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P57-15: Cav-1 AND CYCLIN D1 IN BREAST CANCER PATHOGENESIS

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Here, we investigate the role of caveolin-1 (Cav-1) in breast cancer onset and progression with a focus on epithelial-stromal interactions, that is, the tumor microenvironment. Cav-1 is highly expressed in adipocytes and is abundant in mammary fat pads (stroma), but it remains unknown whether loss of Cav-1 within mammary stromal cells affects the differentiated state of mammary epithelia via paracrine signaling. To address this issue, we characterized the development of the mammary ductal system in Cav-1^{-/-} mice and performed a series of mammary transplant studies using both wild-type and Cav-1^{-/-} mammary fat pads. Cav-1^{-/-} mammary epithelia were hyperproliferative in vivo with dramatic increases in terminal end bud area and mammary ductal thickness as well as increases in bromodeoxyuridine incorporation, extracellular signal-regulated kinase-1/2 hyperactivation, and upregulation of STAT5a and cyclin D1.

Consistent with these findings, loss of Cav-1 dramatically exacerbated mammary lobulo-alveolar hyperplasia in cyclin D1 Tg mice whereas overexpression of Cav-1 caused reversion of this phenotype. Most importantly, Cav-1^{-/-} mammary stromal cells (fat pads) promoted the growth of both normal mammary ductal epithelia and mammary tumor cells.

Thus, Cav-1 expression in both epithelial and stromal cells provides a protective effect against mammary hyperplasia as well as mammary tumorigenesis.

For a recent review, see Sotgia F, Rui H, Bonuccelli G, Mercier I, Pestell RG, Lisanti MP. Caveolin-1, mammary stem cells, and estrogen-dependent breast cancers. *Cancer Res.* 2006 Nov 15;66(22):10647-51.

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P57-16: THE ROLE OF SOLUBLE EPIDERMAL GROWTH FACTOR RECEPTOR ISOFORMS IN REGULATING LIGAND AVAILABILITY AND RECEPTOR ACTIVATION

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The epidermal growth factor (EGF) and receptor (EGFR/ERBB1) families include multiple members, form a complex signaling network, and collectively play fundamental roles in normal mammary gland development and in the pathogenesis of human breast cancer. Recently, there has been an increased focus on the role of EGF-related growth factors in breast cancer as it appears that their expression contributes to resistance to anti-ERBB therapies. EGF domain-containing peptides are synthesized as membrane-bound precursors, which are proteolytically processed to release mature soluble forms of the ligand. However, the mechanisms that regulate ligand availability are not well understood. In this regard, we have identified a soluble EGFR (sEGFR) splice variant, which consists of the entire extracellular domain of the receptor and also contains a unique carboxy-terminal tail sequence.

We have developed an antibody against the sEGFR C-terminal tail sequence and have analyzed normal breast tissue and breast tumors by immunohistochemistry. The anti-sEGFR antibody showed membrane localization in normal breast ducts and reduced staining intensity in breast tumors. In addition, we have transfected a variety of cell lines, with and without endogenous EGFR expression, and have confirmed that sEGFR is membrane localized, using indirect immunofluorescent cell staining, cell fractionation, and membrane-impermeable biotin-labeling techniques. Interestingly, we have shown that sEGFR is associated with the cell surface as a peripheral membrane protein independent of EGFR co-expression.

The role of endogenous sEGFR expression in breast tissues is not known. However, soluble receptors generally act to fine-tune the activities of their ligands and membrane-bound receptors. Cell surface localization of sEGFR provides a direct mechanism for auto-regulation of ligand-receptor interactions. Therefore, we hypothesize that sEGFR isoforms are involved in the processing and availability of EGFR ligands and thereby contribute an additional level of regulation to EGFR family signaling pathways.

Our pilot studies reveal an interaction between sEGFR and the membrane-bound HB-EGF precursor. Additional studies are ongoing to determine whether this association affects growth factor shedding and subsequent activation of EGFR signaling pathways. These studies will test a new mechanism for regulating growth factor availability and may represent an as yet unexplored strategy for the development of novel therapeutic agents targeting EGFR. These novel inhibitors may provide an upstream alternative approach to blocking EGFR family signaling pathways, which could contribute significant inhibitory effect on EGFR and HER2 activity in breast cancer cells.

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P57-17: CELL CYCLE EFFECTS AND MUTAGENICITY OF ETIDRONIC ACID TOWARDS MCF-7 HUMAN BREAST CANCER CELLS

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Cell cycle effects and mutagenicity of etidronic acid (1-hydroxyethane-1,1-diphosphonic acid; 1-hydroxyethylidenediphosphonic acid) (Eti) towards MCF-7 human breast cancer cell line and its multidrug-resistant derivative MCF-7 clone 10.3 cell line were studied in the presence and absence of strontium chloride (Sr). Eti affects calcium metabolism and slows down abnormal bone resorption. The anti-osteolytic activity of this first generation bisphosphonate has led to research on the use of bisphosphonates for the treatment of Paget's disease, osteoporosis, and cancer metastases to the bone. Strontium, which mimics the in vivo behavior of calcium, stimulates bone formation and has been used in the treatment of post-menopausal osteoporosis. There are several reports on anti-tumor effects of bisphosphonates. Clonogenicity assays revealed the cytotoxicity of etidronic acid towards these cell lines, while addition of strontium chloride had no effect. Flow cytometry studies showed that etidronic acid caused a decrease in the S-phase population with concomitant increase in G2/M phase population (Table). Again strontium was without any significant effect. Within the cell cycle, S-phase cells are the most radioresistant while G2/M cells are the most radiosensitive. Therefore the decrease in S-phase population with a corresponding increase in G2/M population would position cells in a more radiosensitive state. Such a shift in cell cycle distribution may be useful if etidronic acid were combined with radioactive strontium (⁸⁹Sr, metastron), which is a beta emitter used in the treatment of bone metastases. For mutagenicity studies, DNA was extracted from MCF-7 cells treated with Eti (10 mM) according to the Q1Amp DNA mini-kit procedure. Amplified PCR products were used to detect p53 mutations using denaturing high pressure liquid chromatography (DHPLC). DHPLC analysis of the conserved region of p53 showed clear alterations in exons 6 and 8 as a result of Eti treatment. It is not clear if these mutations will decrease or increase the chemo or radiation sensitivity of MCF-7 cells.

Treatment	MCF-7		MCF-7/MDR clone 10.3			
	G0/G1(%)	SPhase(%)	G2/M(%)	G0/G1(%)	S-Phase(%)	G2/M(%)
Sr 0.0mM	68.3	25.3	6.4	64.1	18.8	17.1
Sr 3.5mM	70.1	23.7	6.1	67.1	20.1	12.7
Sr 7.0mM	70.1	21.8	7.6	63.3	20.4	16.3
Sr 0.0mM+Eti 0.0mM	69.5	22.6	7.9	67.0	18.5	14.5
Sr 3.5mM+Eti 0.0mM	72.1	21.4	6.5	66.2	18.9	14.9
Sr 7.0mM+Eti 0.0mM	70.8	21.3	7.9	64.6	20.7	14.7
Sr 0.0mM+Eti 10.0mM	63.8	16.9	19.3	70.9	7.2	21.9
Sr 3.5mM+Eti 0.0mM	59.9	17.4	22.7	70.1	7.2	22.7
Sr 7.0mM+Eti 0.0mM	67.1	14.7	18.2	74.1	6.7	18.7

Cell cycle distribution of MCF-7 and MCF-7 clone 10.3 treated with Eti and Sr

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P57-18: SMALL MOLECULE IBR2 TARGETING Rad51 RECOMBINASE FOR PROTEASOME DEGRADATION LEADS TO G1 ARREST AND RETARDS GROWTH OF BREAST CANCER CELLS

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Rad51 recombinase is a key enzyme in homologous recombination for DNA double-strand break (DSBs) repair and DNA replication. Overexpression of Rad51, found in many types of human cancers, contributes to tumor progression and correlates with radio- or chemoresistance, suggesting that Rad51 may serve as a therapeutic target for selectively killing tumor cells. It has been shown that the exogenous BRC repeat of BRCA2 inhibits Rad51 activity both in vivo and in vitro by directly binding to Rad51.

We have identified small molecular inhibitors of Rad51, IBR1 and 2, which target Rad51 and competitively inhibit binding of Rad51 with the BRC repeat and Rad51 multimerization in vitro. Treatment with IBR2 leads to a significant growth retardation of breast cancer cells both in culture and in xenografted tumor model without general toxicity. In IBR2-treated cancer cells, Rad51 was targeted for an accelerated degradation through proteasome pathway, which resulted in cell cycle arrest at G1 phase and subsequent cell death. Overexpression of Rad51 overcame the failure of S phase entry induced by IBR2, whereas depletion of Rad51 by siRNA generated a phenotype similar to IBR2 treatment. These results demonstrate that targeting Rad51 for degradation in breast cancer cells by IBR molecules may have a therapeutic potential.

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FUNCTIONAL STUDY OF BIOLOGICAL MOLECULES II

Poster Session P58

P58-1: GENETIC SCREENS TO IDENTIFY GENES REQUIRED FOR CANCER CELL PROLIFERATION**Stephen Elledge**

Brigham and Women's Hospital

The use of RNA interference (RNAi) to evoke gene silencing in mammalian cell systems using synthetic small interfering RNAs (siRNAs) and expressed short hairpin RNAs (shRNAs) has rapidly become a valuable genetic tool. Here we report the construction and application of an shRNA expression library targeting approximately 25,000 human and 20,000 mouse genes. This library is presently composed of ~100,000 sequence-verified shRNA expression cassettes contained within multi-functional vectors, which permit shRNA cassettes to be packaged in retroviruses, tracked in mixed cell populations via DNA "barcodes." The goal of this library is its ability to inhibit gene function when each vector is present at a single copy. This ability allows one to screen for shRNA expression constructs that are deleterious to cancer cell survival using multiplex screens and microarray deconvolution. This should identify new potential drug targets for cancer therapy. We have applied this library to search for genes required for breast cancer cell survival compared to normal cells. We will report on the success of this strategy and other experiments we have undertaken to identify genes relevant to breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0134 and Howard Hughes Medical Institute.

P58-2: BINDING SPECIFICITY OF INHIBITORY ANTIBODIES TO THE EXTRACELLULAR REGION OF EPIDERMAL GROWTH FACTOR RECEPTOR**Karl R. Schmitz and Kathryn M. Ferguson**

University of Pennsylvania

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases plays a vital role in the regulation of cellular proliferation and differentiation. Aberrant expression and activity of these receptors are associated with epithelial tumor formation, including a significant subset of breast cancers. EGFR activation is initiated by binding of ligand to the receptor extracellular region. Ligand-mediated activation can be abrogated by inhibitory antibodies against the extracellular region, and such inhibitors have proven to be successful anti-cancer therapeutics. Our previous work has shown that some of these antibody-based inhibitors sterically block ligand binding. Additionally, some inhibitors may prevent receptor activation by reducing the flexibility of the extracellular domain, as the extracellular region of EGFR is known to be conformationally dynamic.

Toward a better understanding of and the potential improvement of antibody-based EGFR inhibitors, we have structurally and biochemically characterized the inhibitory activity of several monoclonal, Fab, and camelid antibody fragments. We have employed surface plasmon resonance to investigate inhibitor binding affinities, the ability of inhibitor to compete against ligand for binding to EGFR, and the binding competition between inhibitors for EGFR. These methods have been combined with existing structural information as well as new structural data from X-ray crystallographic studies of inhibitors and inhibitory complexes. Together, these techniques have allowed us to characterize and compare the individual binding surfaces of these inhibitors.

We find that all of the antibody-based EGFR inhibitors in this study interact with domain III of the receptor. A subset of inhibitors binds without overlapping the ligand-binding region of the receptor, and these molecules do not appear to fully abrogate ligand binding. This implies the existence of an additional, allosteric mode of regulation.

These observations further our understanding of EGFR inhibition by antibody-based inhibitors. The structural and biochemical details reported in this work shed new light on the mechanism of receptor activation and may be applicable to the development of improved anti-cancer therapeutics and histological molecular markers.

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P58-3: DEREGLATION OF MATRIPTASE IN BREAST CANCER CELLS**Chen-Yong Lin**

University of Maryland, Baltimore

Matriptase is an epithelial-derived, cell surface serine protease. This protease activates hepatocyte growth factor (HGF) and urokinase plasminogen activator (uPA), two proteins thought to be involved in the growth and motility of cancer cells, particularly carcinomas, and in the vascularization of tumors. Thus, matriptase may play an important role in the progression of carcinomas, such as breast cancer. We examined the regulation of activation of matriptase in human breast cancer cells, in comparison to non-transformed mammary epithelial cells 184A1N4 and MCF-10A. Results clearly indicated that unlike non-transformed mammary epithelial cells, breast cancer cells do not

respond to the known activators of matriptase, serum and sphingosine 1-phosphate (S1P). Similar levels of activated matriptase were detected in breast cancer cells, grown in the presence or absence of S1P. However, up to 5-fold higher levels of activated matriptase were detected in the conditioned media from the cancer cells grown in the absence of serum and S1P, when compared to non-transformed mammary epithelial cells. S1P also induces formation of cortical actin structures in non-transformed cells, but not in breast cancer cells. These results show that in non-transformed cells, S1P induces a rearrangement of the actin cytoskeleton and stimulates proteolytic activity on cell surfaces. In contrast, S1P treatment of breast cancer cells does not activate matriptase, and instead these cells constitutively activate the protease. In addition, breast cancer cells respond differently to S1P in terms of the regulation of actin cytoskeletal structures. Matriptase and its cognate inhibitor, HGF activator inhibitor 1 (HAI-1) colocalize on the cell periphery of breast cancer cells and form stable complexes in the extracellular milieu, suggesting that the inhibitor serves to prevent undesired proteolysis in these cells. Finally, we demonstrate that treatment of T-47D cells with epidermal growth factor (EGF), which promotes cell ruffling, stimulates increased accumulation of activated matriptase at the sites of membrane ruffling, suggesting a possible functional role at these sites.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0252.

P58-4: THE STEROID RECEPTOR RNA ACTIVATOR PROTEIN REGULATES THE ACTIVITY OF TRANSCRIPTION FACTORS**Etienne Leygue, Shilpa Chooniedass-Kothari, Yi Yan, Mohammad Hamedani,****Sophie Carascossa, Yvonne Myal, Stephan Jalaguier, and Vincent Cavailles**

University of Manitoba

Background: Steroid receptor RNA activator gene products consist in a functional RNA (SRA), which increases agonist bound steroid receptor activity, and a protein (SRAP), which exact function remains unexplored. Interestingly, breast cancer patients treated with tamoxifen have a higher probability of survival when their primary tumor expresses detectable levels of SRAP. This suggests that the presence of this protein might characterize a less aggressive tumor phenotype and/or an increase of the sensitivity to tamoxifen. Our overall goal is to investigate SRAP function and elucidate its mechanism of action.

Methods and Results: To investigate its functional role independently of its RNA counterpart, we first have identified SRAP interacting proteins by mass spectrometric analysis of SRAP co-immunoprecipitated samples. Functional gene annotation classification revealed that several of these SRAP interacting proteins are involved in the modulation of transcription. We have determined through protein arrays that SRAP is indeed able to directly interact with various transcription factors including estrogen receptor alpha and beta. Furthermore, we have established that SRAP is associated to chromatin in MCF-7 cells. In light of these observations, we examined the possible effect of SRAP recruitment on transcription sites using the potent GAL4-VP16 hybrid transcription activation system. We observed that SRA possesses a transcriptional repressive activity capable of inhibiting the GAL4-VP16 transcription activity. This SRAP transcriptional repressive potential is sensitive to trichostatin A (a HDAC inhibitor) treatment suggesting an involvement of HDACs in SRAP mechanism of action. In support of this hypothesis, we determined that SRAP is able to co-immunoprecipitate HDAC activity. Interestingly, we also observed, by transiently introducing luciferase-reporters driven by different estrogen receptor elements (EREs) that SRAP could, in HeLa cells, also activate the agonist- or antagonist-bound activity of the estrogen receptor in a ERE dependent manner.

Conclusions and Relevance: Together our results indicate that similarly to SRA RNA, SRAP interacts with transcriptional regulators and is involved in the modulation of transcription. SRAP, as its RNA counterpart, can positively regulate the activity of estrogen receptor when assessed in specific reporter systems. However, unlike SRA RNA, SRAP is also capable of negatively regulating transcription through the recruitment of HDAC activity. Identifying endogenous genes sensitive to the positive and negative SRAP effects will be our next step in dissecting its mechanisms of action. Understanding the mechanisms involved in the action of the products of the bi-faceted SRA/SRAP system will lead us to propose new therapeutic strategies to fight breast cancer.

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P58-5: ROLE OF HISTONE DEMETHYLASE JARID1B IN BREAST CANCER**Qin Yan, Jiayun Liu, and William G. Kaelin**

Dana-Farber Cancer Institute

The *JARID1B/PLU-1* gene is highly expressed in primary breast cancers and breast cancer cell lines but not in normal adult breast tissue. The ideal targets for cancer therapy are enzymes required for proliferation of cancer cells but not for proliferation of

normal cells. In this regard, JARID1B is such an attractive candidate but has not been well studied. JARID1B was recently found to be a histone demethylase for tri-methylated histone H3K4, the epigenetic marker for active gene expression. To determine whether JARID1B upregulation is important for breast cancer formation, MCF7 breast cancer cell lines with stable knockdown of JARID1B have been generated. These cell lines will be assessed for their ability to form tumors in mouse xenograft experiments. The role of JARID1B histone demethylase activity in tumor formation will also be investigated. These studies would shed light on the functions of JARID1B in carcinogenesis and could serve to validate JARID1B as a target for therapeutic intervention in breast cancer.

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P58-6: DISCOVERY AND ROLE OF A NOVEL EICOSANOID FAMILY IN BREAST CANCER

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There is strong evidence for the induction of the pro-inflammatory enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in many types of cancer, including breast cancer. Upregulation of both enzymes is associated with promoting tumorigenesis and a negative prognosis of the disease. The 5-LOX (leukotriene) and COX-2 (prostaglandin) pathways have traditionally been viewed as independent biosynthetic routes to eicosanoid lipid hormones since the committed step toward either pathway is taken as the initial oxygenation of the common substrate, arachidonic acid. A cross-over interaction of the two pathways is an unexplored possibility, but it is at the focus of this research project. Our recent enzymological analyses established that the 5-LOX product, 5-hydroxy-eicosatetraenoic acid (5-HETE), is an excellent COX-2 substrate *in vitro* (Schneider C. et al., *J. Am. Chem. Soc.* 128 [2006], 720). Using liquid chromatography-mass spectrometry and nuclear magnetic resonance techniques, we have identified the product of the COX-2 oxygenation of 5-HETE as a novel double endoperoxide derivative of arachidonic acid. This product has the potential to evolve into a novel family of biomediators. In work in progress, we are now trying to provide evidence that the cross-over of the 5-LOX and COX-2 pathways is a functional possibility *in vivo*. We are currently testing the hypothesis that breast cancer cells co-expressing 5-LOX and COX-2 form a novel series of eicosanoid metabolites in a cross-pathway interaction and that these metabolites regulate proliferation and differentiation of the cancer cells and the surrounding neoplastic tissue. The concept of a cross-over of the leukotriene and prostaglandin inflammatory pathways could ultimately lead to a novel strategy of manipulating endogenous small molecules for the treatment of breast cancer using established anti-inflammatory medications.

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P58-7: AUTOCRINE AND PARACRINE EFFECTS OF pBP1, A HOMEOTIC PROTEIN, IN BREAST CANCER CELLS

Patricia Berg,¹ Jinguen Rhee,² Louis Bivona,² Yan-Gao Man,³ and Kristen Baxter²

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Background and Objectives: BP1 is a member of the homeobox gene family, a highly conserved family of transcription factor genes. pBP1 is a homeotic protein that is upregulated in 80% of invasive ductal breast tumors. We have previously shown that the percentage of pBP1-positive breast cancer cases increases with the extent of cellular proliferation and carcinogenesis. BP1 expression is associated with aggressive tumors: 100% of ER-negative tumors were BP1 positive compared with 73% of ER-positive tumors. Interestingly, pBP1 is cytoplasmically located in breast cancer cell lines and in tumors. Homeotic proteins can be secreted and internalized although this process has not been examined in cancer until now. Moreover, the functions of internalized homeoproteins are unknown. The objectives of this study were to determine whether pBP1 is secreted by breast cancer cells and, if so, to determine its function(s).

Methods: Secretion of pBP1 was tested by collecting and concentrating conditioned media (CM) from cell lines and performing western blot analysis. Mitogenic effects of secreted pBP1 were tested by incubating five cell lines with 10X CM from MCF-7 cells engineered to overexpress pBP1 and assessing growth by MTT assays. As a control, pBP1 was immunoprecipitated from 10X CM, and cells were grown in this depleted media (DM). Expression of selected genes was tested by QRT-PCR in breast cancer and normal cells after growth in 10X CM.

Results: pBP1 is secreted by MCF-7 cells, T47D cells, and MDA-MB-231 breast cancer cells but not by MCF10A or H16N2 normal breast epithelial cells. To test the generality of this effect, we also demonstrated secretion of the homeoprotein HOXB7 by

the same breast cancer cell lines. Internalization of pBP1 was demonstrated using chambers; donor cells secreting pBP1 were grown in the top chamber and recipient cells expressing low pBP1 levels in the bottom chamber. Immunostaining the recipient cells revealed increased pBP1 positivity. CM was obtained from MCF-7/O2 cells, a cell line that overexpresses pBP1. MCF-7, T47D, MDA-MB-231, MCF10A, and H16N2 cells were grown in 10X CM, and stimulation of growth was observed in all five cell lines. However, there was no growth stimulation of cells grown in CM from MCF10A cells or grown in depleted media. These data suggest that mitogenic effects of pBP1 were due to pBP1 alone or in combination with other secreted proteins. Breast cancer cells that have internalized pBP1 show upregulation of several genes, including bcl-2 and Twist.

Conclusions: Our data indicate that pBP1 and another homeotic protein, pHOXB7, are secreted by breast cancer cells. This is a new phenomenon in breast cancer, suggesting that homeotic transcription factors in general may be present in the blood of breast cancer patients and thus amenable to detection and targeting. These secreted proteins may be useful in early detection, prognosis, and monitoring therapy. Importantly, internalization of pBP1 stimulates growth of recipient cells in either an autocrine or paracrine manner. This effect may be related to the aggressiveness of BP1-positive tumors.

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P58-8: ABSTRACT WITHDRAWN

P58-9: NOVEL ACTIVITY FOR SNAIL IN INTEGRIN REGULATION

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Background and Objectives: Snail is expressed in node-positive, but not in low-grade, node-negative ductal breast carcinomas, suggesting its importance for tumor metastatic progression [1]. Interestingly, consensus binding sites for Snail family members have been identified in the beta1 (β) integrin promoter. Moreover, Slug, a Snail relative, impedes β 1 transcription in keratinocytes [2]. Based on these findings, we postulate that Snail reduces β 1 integrin transcription in metastatic ductal breast carcinoma. We predict that by reducing β 1 integrin expression levels, Snail decreases tumor cell adhesion to matrix proteins. This prediction is based on the knowledge that the adhesive strength of a cell is determined in part by the number of integrins on the cell surface. Furthermore, we postulate that by reducing tumor cell adhesive strength, Snail promotes breast tumor cell migration. This prediction is based on the fact that cell migration is dependent on a cell's attainment of an intermediate adhesive strength to extracellular matrix with either too weak or too strong cell adhesion being inhibitory for migration [3,4]. Of note, Slug, a Snail family member, reduces but does not abolish β 1 integrin transcription in keratinocytes [2]. Similarly, we postulate that Snail reduces, but does not eliminate β 1 integrin expression in breast tumors. We predict that by maintaining reduced levels of β 1 integrin on metastatic breast tumor cells, Snail imparts an intermediate adhesive strength supportive of cell migration.

Methodologies: Breast tumor cell lines were transfected with either a Snail-specific small interfering RNA or a Snail expression vector. β 1 integrin levels were measured by reverse transcription-PCR, immunoblotting, and flow cytometry. The effect of altering Snail expression in breast tumor cell lines on β 1 integrin-dependent adhesion and cell migration was also examined.

Results to Date: Reducing Snail expression in breast tumor cells increased β 1 integrin mRNA and protein levels and increased cell adhesion to β 1-binding matrix proteins. Conversely, the transfection of cells with exogenous Snail decreased β 1 integrin mRNA and protein levels and reduced cell adhesion. We are currently investigating if the ability of Snail to reduce β 1 integrin expression relates to its suppression of β 1 integrin transcription. In addition, we are examining the impact of this Snail activity on tumor cell migration.

Conclusions: These studies define a novel activity for Snail (reducing β 1 integrin transcription) in breast carcinoma. We show that this Snail activity decreases the adhesive strength of breast tumor cells to β 1 integrin-binding matrix proteins. Based on the knowledge that cell migration is dependent on a cell's attainment of an intermediate adhesive strength, we are currently investigating the hypothesis that Snail promotes breast tumor cell migration by maintaining reduced β 1 integrin levels. Since tumor cell motility is a requirement for metastasis, we predict that this Snail activity is relevant to tumor metastatic progression, a topic for future studies.

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P58-10: ABSENCE OF HIGH FREQUENCY SELADIN-1 MUTATIONS IN BREAST CANCER

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Seladin-1 has been described as a novel regulator of p53, a major human tumor suppressor gene (TSG) [1]. Seladin-1 acts to counteract Mdm2 and promote accumulation of p53 in response to oncogenes and oxidative stress [1]. p53 is implicated in breast cancer progression, but it is mutated only in a fraction of human breast tumors [2], suggesting that additional mechanisms might be involved in functional inactivation of the p53 checkpoint in tumors expressing wild-type p53. To determine if Seladin-1 is a TSG involved in breast cancer we proposed to: (1) Identify possible Seladin-1 mutations in primary breast tumors; (2) investigate the alterations of Seladin-1 expression in breast cancer cells; (3) perform functional assays on tumor-specific Seladin-1 mutants.

We amplified Seladin-1 exons from 60 breast tumor genomic DNA samples (obtained from a commercial source (Oncomatrix, Inc.), followed by the sequence analysis of the open reading frames. As a result, we did not encounter variations in the coding region and exon-intron junctions (other than known single nucleotide polymorphisms (SNPs)). A single new high frequency insertion/deletion polymorphism was found in the non-coding region. In cases showing this particular deletion/insertion SNP, both tumor material and patients DNA obtained from unaffected tissues had the same polymorphism, suggesting that it is not associated with the spontaneous breast cancer. We isolated RNAs from human breast cell lines MCF7, BTK20, T47D, HBL100, MDA-MB-231, MDA-MB-468, and MCF10A. Seladin-1 probe labeling and hybridization were done using commercial kits. As a control, we used RNA from three different batches of normal mammary epithelial cells (HMEC).

When RNAs from 10 primary breast cancer cell lines were analyzed, the conclusions on Seladin-1 gene expression was that this gene is usually abundantly expressed in breast cancer cells (with the exception of MDA-MB-231). Interestingly, expression of Seladin-1 in MDA-MB-231 cell line was subjected to regulation by 5aza-2'-deoxycytidine (5AzaDC), a demethylating agent. An increase in Seladin-1 expression in response to 5AzaDC indicates that this putative cell line was a candidate for promoter methylation studies using methylation-specific PCR and bisulfite sequencing.

Since we did not detect breast tumor-specific Seladin-1 mutants (in the coding region), we concentrated on wild-type Seladin-1 function and found that Seladin-1 had inhibitory effect on growth of some breast cancer cells (notably MDA-MB-231) expressing it at the low levels, while having no effect on T47D or MDA-MB-468 that express Seladin-1 at much higher levels. The work on characterizing the inhibitory function of Seladin-1 in breast tumor cells is currently ongoing.

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P58-11: GLUCOCORTICOID-DEPENDENT REGULATION OF TUMOR-DERIVED TOLEROGENTIC TRANSCRIPTION FACTOR FOXP3: ROLE IN TUMOR IMMUNO-RESISTANCE TO APOPTOSIS IN BREAST CANCER

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Immunotherapy of breast cancer has been established as a novel alternative to conventional therapy in the form of passive immunotherapeutic strategies such as adoptive transfer of antigen-specific T lymphocytes or the infusion of monoclonal antibodies specific for a tumor associated antigen (TAA), and active immunization with several modalities of tumor vaccines. However, the effectiveness of either therapeutic approach is significantly impaired by the persistence of breast carcinoma cells with elevated resistance to programmed cell death (apoptosis) and/or the acquired tumor immune tolerance. FOXP3 is a transcriptional regulator exhibiting a critically important role in the development and function of the regulatory T cells (Tregs) and the consequent definition of immune tolerance. It has been shown that expression of FOXP3 in tolerogenic environments correlates with increased resistance to apoptosis in Tregs by the regulation of apoptosis-related genes such as Bcl-2. Recently, we have identified endogenous expression of FOXP3 in several mouse and human breast carcinoma cell lines. Analysis of the 5'-regulatory region of the human FOXP3 gene revealed multiple

putative glucocorticoid responsive elements (GRE) in addition to other nuclear receptors and core transcriptional regulators. Thus, we hypothesize that this milieu of steroid hormone receptor activities control the expression of FOXP3 in human breast cancer cells and regulate the tumor-derived immune tolerance and resistance to apoptosis (Survival). To test this hypothesis, first we examined the expression pattern of FOXP3 in various human breast carcinoma cell lines (MCF-7, BT-474, SK-BR3, and ZR-75), observing a direct correlation between the status of expression of estrogen receptor (ER) and the expression of tumor-derived FOXP3. Secondly, we demonstrated the responsiveness of FOXP3 mRNA expression in cultured MCF-7 cells to the GR agonist, dexamethasone (Dex) and to the selective GR antagonist, mifepristone (RU-486). Our results indicate that Dex is able to increase the expression of FOXP3 in MCF-7 and this effect is abrogated by the use of RU-486, suggesting the partial role of GR in the transcriptional control of FOXP3 expression in human breast cancer cells. In addition, we demonstrated that nitric oxide (NO) suppresses the expression of FOXP3 by interfering with the GR/ER-dependent transcriptional activation of FOXP3 promoter. Further, we evaluated the relative in vitro sensitivity of cultured human breast carcinoma cell lines to receptor-mediated apoptosis (Fas, TNF- α , and TRAIL) resulting in a direct correlation between the status of expression of FOXP3 and their resistance to TNF- α and TRAIL-mediated apoptosis. In addition, we examined the differential profile of cytokine gene expression by focus cDNA microarray analysis of mRNA populations from breast carcinoma cells treated in the presence or absence of RU486 resulting in the differential expression of TGF- β , IL-10 and IL-6 correlating with FOXP3 expression. Collectively, our results demonstrate the role of FOXP3 expression by breast cancer cell in conferring tumor resistance to receptor-mediated apoptosis and providing potential means to target the expression of FOXP3—such as the use of GR antagonists—to sensitize breast carcinoma cells to apoptosis and remove immunosuppressive factors, breaking tolerance and promoting a more effective tumor-specific immunotherapy.

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P58-12: IDENTIFICATION OF MESOTRYPSIN AS A SERINE PROTEASE INVOLVED IN BREAST CANCER PROGRESSION AND POTENTIAL THERAPEUTIC TARGET

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Background and Objectives: Extracellular proteases are associated with nearly every aspect of breast cancer development – proliferation, invasion, angiogenesis, and metastasis – and have received attention as potential therapeutic targets. While most attention has focused on the matrix metalloproteinases (MMPs) as therapeutic targets, the serine proteases play key roles in many of the same tumorigenic processes and may pose promising targets for treatment of breast cancer. Using a physiologically relevant, 3-dimensional culture system modeling early stages of human breast cancer progression, we discovered that treatment of HMT-3522 T4-2 breast cancer cells with the broad-spectrum serine protease inhibitor aprotinin could suppress the malignant phenotype. We hypothesized that specific inhibition of one or more secreted or membrane-associated serine proteases blocked the malignant phenotype in our breast cancer model and that this protease or group of proteases might represent a novel and promising target for development of highly selective molecularly targeted therapeutics for breast cancer treatment. We proposed to identify those serine proteases for which upregulation correlated with malignancy in the HMT-3522 breast cancer progression series.

Methods: A panel of candidate proteases was screened for expression levels in malignant T4-2 cells relative to nonmalignant precursor S1 cells, both grown under physiologically relevant 3D conditions, using real-time quantitative RT/PCR techniques. For candidate proteases found to be upregulated with cancer progression, specific inhibition of expression by RNA interference was evaluated, looking for evidence of suppression of the malignant growth phenotype of T4-2 cells in 3D culture. Treatment of T4-2 knockdown cells in 3D culture with purified recombinant protease was evaluated for restoration of the malignant growth phenotype.

Results: We discovered five serine proteases significantly upregulated in T4-2 cells relative to S1 cells: mesotrypsin, prostasin, transmembrane protease serine 3, neutrotypsin, and matrilysin-1. Among these, knockdown of mesotrypsin (PRSS3) in T4-2 cells led to an attenuation of the malignant growth phenotype. Treatment of these cells with purified recombinant mesotrypsin restored the malignant growth phenotype.

Conclusions: We have identified a serine protease, mesotrypsin, which is upregulated in a physiologically relevant, three-dimensional cell culture model of breast cancer progression. We have established that mesotrypsin plays a functional role in promoting malignant growth and have determined that specific inhibition of the expression of this enzyme attenuates the 3D malignant growth phenotype in our breast cancer model. Mesotrypsin has not been associated previously with breast cancer, but now presents a potential novel target for the development of new molecularly targeted therapies for breast cancer.

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P58-13: IDENTIFICATION OF MICRORNA-21 TARGET GENES BY A GENETIC SELECTION SYSTEM

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Background: As naturally occurring small non-coding RNAs, microRNAs (miRNAs) have been recently shown to play a key role in human malignancies, in particular breast cancer because they are capable of regulating expression of protein coding genes. It is believed that miRNAs exert their silencing function by targeting the 3'-untranslated regions (3'-UTRs) of mRNAs. Thus, identification of miRNA target genes is a critical step to understand the underlying mechanism of miRNA-mediated oncogenesis. Currently, this largely relies on computer-aided algorithms. Since the mechanism governing the miRNA targeting specificity in the cell is not fully understood, the computer-aided predictions are still not able to provide a precise picture of miRNA regulatory networks and need further in vivo validations. Hence, the objective of this study is to develop a biological system for identification of miRNA target genes.

Methods: We focused on *mir-21* in this study because our previous study indicates that *mir-21* is overexpressed in breast cancer, and further study shows that *mir-21* plays an oncogenic role. Thus, we constructed a selection plasmid that carried a transcriptional repressor fusion gene tTR-KRAB. In addition, this selection plasmid carried the puromycin resistance gene (Pu) coding for puromycin-N-acetyl-transferase under the tet operator (tetO). We then cloned cDNAs carrying the 3'-UTRs from a breast tumor cDNA library into the downstream of tTR-KRAB. The principle of the selection is as follows. Under a normal condition, cells transfected with this plasmid are killed in the presence of puromycin because Pu is repressed by tTR-KRAB. However, when the same plasmid carries an interaction site for *mir-21* in the 3'-UTR of an mRNA along with *mir-21* expression vector, the cells survive because suppression of tTR-KRAB leads to expression of Pu. Therefore, only the cells that carry a putative *mir-21* target are selected out. Thereafter, the genomic DNA was isolated from the survival colonies, and PCR reaction was carried out to isolate potential *mir-21*-regulated clones using specific primers derived from the selection plasmid. Further confirmation was performed by luciferase assays and DNA sequencing.

Results: To validate our system, we used a known *mir-21* target tropomyosin 1 (TPM1) that was previously identified by a proteomic approach in our laboratory. We demonstrated that the selection plasmid carrying the TPM1 3'-UTR along with the *mir-21* expression vector caused reduction of tTR-KRAB protein and substantially increased the number of survival colonies. Using this system, we identified several putative targets for *mir-21*, such as programmed cell death 4 (PDCD4), maspin, and cytokeratin 8. Of interest, both PDCD4 and maspin, like TPM1, are known tumor suppressor genes that play a role in tumor cell invasion and metastasis. Moreover, they each also carried a conserved *mir-21* binding site. Using a luciferase reporter system, we demonstrated that the *mir-21* binding site in the 3'-UTR was critical for *mir-21* regulation. Finally, we showed that overexpression of both PDCD4 and maspin increased invasiveness in the metastatic breast MDA-MB-231 cells.

Conclusion: This is a simple and rapid selection system for identification of *mir-21* targets, which does not only provide a complementary approach to the in silico methods, but also could serve as a platform for targets of other miRNAs.

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P58-14: ABSTRACT WITHDRAWN

P58-15: FILLING IN THE GAP IN GALECTIN-1 EXPORT

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Background and Objectives: Many eukaryotic secreted proteins generally contain a leader- or export sequence, which directs their synthesis to the endoplasmic reticulum, where they are then trafficked to the Golgi and transport vesicles, to fuse with the plasma membrane in releasing their content to the extracellular space. Interestingly, galectins, fibroblast growth factors (FGF) 1 and 2, interleukin (IL) 1b, the HIV-1 transactivating protein TAT, and the herpes virus structural protein VP22 are a few examples of proteins that do not contain leader sequence. How these leader-less proteins are secreted is of special importance because of their roles in a wide range of biological processes and pathogenesis. Because the extracellular galectins exhibit important roles in cancer as these molecules contribute to neoplastic transformation, tumor cell survival, angiogenesis, tumor metastasis, modulation of immune and inflammatory responses, and finally, in helping tumors to escape immune surveillance by the cytotoxic tumor infiltrating T cells, we proposed to study the mechanism by which galectin-1, a prototype for all galectins, is secreted by cancer cells.

Brief Description of Methodologies: The primary method to identify proteins that interact with galectin-1 was the yeast two-hybrid analysis. The Match Maker 2-Hybrid System 3 kit from Clontech, CA, was utilized. A cDNA coding the N-terminal 1-40

amino acids of galectin-1 was subcloned into pGBKT7 DB domain and used as bait, and then the mammary gland cDNA library was screened (Clontech, CA) for interacting proteins. The RT-PCR, immunoblotting, and immunocytochemistry were used to characterize the galectin expression in different cancer cell lines.

Results to Date: This yeast two-hybrid search identified several proteins interestingly known to be involved in the secretory pathway. Among the proteins that might have roles in the protein trafficking are, adaptor-related protein complex 1, gamma 2 subunit (AP1G2) variant 2, epsin 2, and translocon-delta subunit. In addition, this two-hybrid analysis indicates that galectin interacts with the N- and C-termini of P-glycoprotein (Pgp). To better understand the galectin secretion, we then analyzed the galectin expression levels in different cancer cell lines. These studies have identified several cancer cell lines as potential model systems for studying the mechanism of galectin secretion and the functional roles of the interactors in galectin-1 secretion. Many of these cancer cells secrete galectin-1 into the extracellular milieu, which is also bound to an unknown receptor on the cell surface.

Conclusions: Adaptor proteins including AP1G2 and epsin are important components of clathrin-coated vesicles, transporting ligand-receptor complexes from the plasma membrane or from the trans-Golgi network to lysosomes/plasma membrane. The translocon subunit delta is a component of translocon, a channel through which cotranslational trafficking of nascent polypeptides takes place. Interaction with Pgp suggests this drug pump may extrude galectin-1. These observations suggest that galectin-1 secretion may take two routes, one is through the conventional secretory path, and the other is through P-glycoprotein-mediated efflux.

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P58-16: IDENTIFICATION AND BIOLOGICAL CHARACTERIZATION OF A SMALL-MOLECULE ANTAGONIST OF RhoC GTPase

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RhoC GTPase belongs to the Ras superfamily of small GTPases. In its GTP-bound, activated form, RhoC specifically interacts with its effector proteins to elicit downstream signaling. RhoC overexpression is detected in over 80% of the tumors of patients with inflammatory breast cancer (IBC), the most lethal form of breast cancer, but not in normal breast cells. At present, there is no specific compound available for the treatment of IBC. Blocking RhoC-effector interaction using a cell-permeable, non-peptide small molecule is therefore an attractive therapeutic approach. We have conducted a RhoC structure-based search and identified a small-molecule inhibitor RhEDC-2, which specifically interferes with the interactions between RhoC and its effector proteins in a mammalian two-hybrid analysis. In contrast, this compound had little effect on disrupting RhoA-effector interactions. RhEDC-2 significantly induced IBC cell apoptosis in a dose-dependent manner with IC₅₀ around 1 μM. When treated with 3 μM of RhEDC-2 for 24 hours, IBC cells ceased proliferation. We have confirmed that RhEDC-2 functions to specifically target RhoC since it induced a significantly higher apoptosis index in RhoC-overexpressing breast cells. Taken together, RhEDC-2 is able to specifically interfere with RhoC function in IBC cells and serves as a potential lead compound for anti-IBC drug development.

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P58-17: A NOVEL CLATHRIN COAT COMPLEX SUGGESTED FOR ENDOCYTIC RECYCLING OF ErbB2

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Increased ErbB2 on the surface of mammary epithelial cells enhances mitogenic signaling, and this process has been implicated as a major cause of breast cancer. Recent evidence suggests that endocytic recycling contributes to the high surface level of ErbB2. However, unlike all other currently known intracellular transport pathways, the conventional mechanism of protein sorting by coat proteins is thought not to play a significant role in endocytic recycling. Reversing this prevailing view, we have recently identified a novel clathrin coat complex for endocytic recycling. Applying this insight, we have found that a fraction of ErbB2 colocalizes with components of this novel coat complex in endosomal compartments. Studies are now under way to examine whether perturbing the function of this coat complex reduces the level of surface ErbB2 and thereby reducing mitogenic signaling.

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P58-18: hEcd, A NOVEL REGULATOR OF MAMMARY EPITHELIAL CELL SURVIVALVimla Band,¹ Channabasavaiah B. Gurumurthy,¹ Jun Hyun Kim,¹ Ying Zhang,² and Hamid Band¹¹University of Nebraska Medical Center and ²Evanston Northwestern Healthcare Research Institute

Proliferation of normal mammary epithelial cells (hMECs) is tightly regulated by the availability of stimulatory and inhibitory factors as well as by their intrinsic replicative life span; most cells in our organs perhaps with the exception of stem cells go through a limited number of replicative cycles prior to differentiation, senescence, or death. Together, these controls represent critical barriers to oncogenic transformation, and these barriers must be overcome to propel normal cells into continued cycles of proliferation, a state of immortalization. Continuous replication renders immortal cells susceptible to accumulation of further genetic alterations that promote full malignant transformation.

We have identified a novel protein using the yeast two hybrid analysis with human papilloma virus oncogene E6 (the most efficient oncogene to immortalize hMECs in vitro), designated as hEcd (human orthologue of *Drosophila ecdysoneless*), as a novel regulator of hMEC survival. We have recently shown that the hEcd protein binds to and stabilizes the p53 protein, and its overexpression in mammalian cells enhances the p53 target gene transcription whereas its knockdown has the opposite effect. However, unlike stable knockdown of p53 (which extended the lifespan of normally senescent epithelial cells), stable hEcd knockdown led to dramatic decrease in cell viability in both p53-expressing and nonexpressing hMECs, suggesting a novel p53-independent role for hEcd in hMEC cell survival. Consistent with this notion, deletion or mutations of yeast or *Drosophila Ecd* proteins also reveals a cell autonomous role in cell survival through mechanisms that remain to be delineated. Importantly, hEcd is overexpressed in breast cancer cell lines and tissue specimens. Based on these data emanating from yeast, *Drosophila*, and hMEC systems, we hypothesize that hEcd is a novel cell survival protein and contributes to oncogenic transformation. Defining novel survival pathways that are obvious targets for cancer therapy will help in future design of novel therapies for breast cancer.

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P58-19: DISSECTING KEY DETERMINANTS OF BREAST EPITHELIAL ARCHITECTURE

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Breast epithelial cell contact with the basement membrane induces signals for growth inhibition, cytoskeletal alterations, polarity, and tissue-specific gene expression. Just as correct cell-basement membrane interactions are critical to normal epithelial architecture and function, alterations in these signaling mechanisms are key determinants of cancer progression. To understand the alterations in cell-basement membrane interactions that contribute to cancer progression and metastasis, it is necessary to understand, in detail, the mechanisms of normal cell response to the basement membrane.

To dissect the roles of specific basement receptors in mammary gland biology, we are examining the architecture and function of mammary epithelial cells in vivo after genetically eliminating the expression of known basement membrane receptors, which include members of the integrin receptor family and dystroglycan. The elimination of integrin and dystroglycan gene expression from functionally normal mammary epithelial cells is being achieved by conditional gene knockout in transgenic mice, using Cre-Lox recombination, where the Cre recombinase induces DNA recombination between loxP sites flanking the target gene. In our study, Cre recombinase expression is driven in transgenic mice by the mammary-specific promoter of the mouse mammary tumor virus (MMTV), inducing a mammary-specific knockout in vivo. We first determined the consequence of beta1 integrin deletion using the MMTV promoter and showed that normal tissue architecture develops though mid-pregnancy, but a failure of differentiation occurs later in pregnancy, leading to a lactation deficiency in more than 90% of the mice. These results confirm and expand results from laboratories that used other mammary-specific promoters to drive Cre expression and showed that genetic deletion of the beta1 integrin family from mammary epithelial cells did not dramatically alter tissue architecture. We next demonstrated that the genetic deletion of dystroglycan by MMTV-Cre expression produced an even milder phenotype, with minor hyperplasia of the epithelium exhibited at mid-pregnancy, but no prominent lactation deficiency at later stages. Functional compensation between dystroglycan and integrins could explain the relatively minor effects on tissue architecture induced by these individual gene deletions. However, remarkably, the simultaneous deletion of both dystroglycan and beta1 integrins produced no greater defect in mammary gland architecture and function than did the deletion of either gene alone.

From these results, we conclude that, in addition to dystroglycan and beta1 integrin signaling, there likely exists undiscovered cell-basement membrane interactions that are key determinants of breast epithelial architecture. We propose that the functions of these novel basement membrane receptors are obscured to investigators by the overlapping function of known basement membrane receptors. We plan to expand on this genetic approach to reveal novel receptor functions and to open new avenues of investigation into the biology of normal and tumorigenic breast epithelial cells.

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GENETIC RISK

Poster Session P59

P59-1: IDENTIFICATION OF GENES AFFECTING BREAST CANCER DEVELOPMENT

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The early detection of breast cancer is very important for treatment and prognosis. The mechanisms underlying the progression and growth from mammary hyperplasias to tumors are poorly understood. The *Apc^{Min/+}* mouse is a well-characterized model to address the genetic control of mammary tumor development, which is affected by the different genetic background. Genetic analysis of tumor development in a backcross of (FVB.B6*Apc^{Min/+}*) x B6 *Apc^{Min/+}* mice has identified a modifier on chromosome 9 that significantly affects tumor multiplicity and a modifier on chromosome 4 that significantly affects tumor latency and affects tumor number with suggestive significance. This modifier was also identified in a backcross involving 129X1/SvJ and B6 *Apc^{Min/+}* mice. A modifier on chromosome 18 specifically affects tumor latency but not tumor number. Kaplan-Meier analysis suggests there is at least an additive interaction affecting tumor latency between the loci on chromosomes 4 and 18. We also identified a modifier locus on chromosome 6 that interacts with the loci on chromosome 4 and chromosome 9 to affect tumor number. These results suggest that multiple genetic loci control different aspects of mammary tumor development. None of these modifiers is associated with intestinal tumor susceptibility, which indicates that these modifiers act on tumor development in a tissue-specific manner.

To assess the combinational effect of mammary modifiers, *Apc^{Min/+}* was transferred into the FVB background. FVB.B6-*Apc^{Min/+}* mice develop mammary tumors with a much longer latency after ENU treatment and have similar susceptibility to mammary hyperplasias and tumors as the (FVBx B6) F1 *Apc^{Min/+}* mice. Interestingly, we found the genetic background could also affect the histological types of mammary tumor. The B6-*Apc^{Min/+}* mice develop mammary squamous cell carcinomas while the FVB.B6-*Apc^{Min/+}* have mammary adenocarcinomas. Immunohistochemistry (IHC) analysis revealed nuclear β -catenin accumulation in mammary hyperplasias and squamous metaplasias, suggesting mutations on *Apc* are the early essential steps for both tumor types. Nuclear β -catenin is lost during the progression from mammary hyperplasias to adenocarcinomas in FVB.B6-*Apc^{Min/+}* females. We also characterized these two tumor types on E-cadherin and estrogen receptor expression. The membrane E-cadherin/ β -catenin is differentially expressed in mammary squamous cell carcinomas and adenocarcinomas. ER- α is also lost during the progression from hyperplasias to adenocarcinomas. This information would be the first step to understand the decision of mammary tumor types.

To understand the molecular mechanisms affecting mammary tumor development, we focused on the modifier *Mmom2*. The N10 congenic mice analysis confirmed the existence of *Mmom2* and narrowed the modifier into a 12 Mb region. Further work is going on to identify the gene/genes of modifier *Mmom2*.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0413.

P59-2: DO STRUCTURAL MISSENSE VARIANTS IN THE ATM GENE FOUND IN WOMEN

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Background and Objectives: Ataxia telangiectasia, or A-T, is an autosomal recessive, pleiotropic hereditary disorder characterized by progressive cerebellar degeneration, immunodeficiency, telangiectasia, extreme sensitivity to ionizing radiation, and a predisposition to cancer development. Mutations causing A-T were mapped to chromosome 11q22-23, and the gene mutated in A-T, called ATM for ataxia telangiectasia-mutated, was eventually identified by positional cloning.

Epidemiologic studies of families of A-T patients have demonstrated an increased risk of breast cancer in females. Based on epidemiological data, an estimated 13% of women with breast cancer may have a 30% or more lifetime risk of breast cancer [Sommer et al., 2003], but direct causative evidence is lacking. The central hypothesis of this study is that the knock-in mice with ATM structural variants will have a high risk of breast cancer. These variants are found in humans and are insufficient to cause A-T. If confirmed, this would be the first direct causal demonstration that non-A-T-causative missense structural variants of the ATM gene can cause breast cancer. A second hypothesis is that women with ATM structural variants are predisposed to other kinds of cancers as well. If ATM structural variants can cause breast cancer, the mouse models generated herein can be used to test preventive, chemopreventive, and therapeutic interventions. The objective of this study is to construct ATM knock-in mouse models to investigate the relationship between ATM missense mutations and cancer, with emphasis on breast cancer.

Methods: (1) Constructing knock-in targeting vectors, (2) PCR screening of recombinant clones, (3) southern screening of ES cells, (4) expansion and microinjection of positive ES cell clones into blastocysts, implantation and birth of chimeras, (5) housing and breeding of chimeras to F1 heterozygotes and confirmation of germline transmission via genotyping, (6) breeding to produce two mouse knock-in ATM lines, as well as

positive (ATM SRI) and negative (ATM trunc) control lines, and (7) determining the rate of breast and other cancers in the mouse lines and in wild-type controls.

Results:

1. Two constructions of knock-in targeting vectors for ATM knock-in I (exon 50, 7327 A>G, AAA>GAA, lys2443Glu) and ATM knock-in II (exon 11, 1877 T>G, TTT>TGT phe626cys) have been established.
2. Recombinant embryonic stem cells clones have been generated: Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of i7L IC1 C57BL/6 embryonic stem cells. After selection in G418 antibiotic, surviving clones were expanded. Recombinant ES clones were identified by southern and PCR analysis.
3. Microinjection of positive ES cell clones into blastocysts and implantation and birth of multiple chimeras were achieved.

Reference:

Sommer SS, Jiang Z, Feng J, Buzin CH, Zheng J, Longmate J, Jung M, Moulds J, Dritschilo A. 2003. ATM missense mutations are frequent in patients with breast cancer. *Cancer Genetics and Cytogenetics* 145:115-120.

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P59-3: CHEK2 MUTATIONS IN AFRICAN AMERICAN WOMEN WITH BREAST CANCER—RESULTS OF A BREAST CANCER FAMILY REGISTRY STUDY

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CHEK2, encoded on chromosome 22q, is a protein kinase that is involved in cell-cycle checkpoint control by causing arrest in G1 and G2 after DNA damage. The protein-truncating CHEK21100delC mutation abolishes the protein kinase activity of CHEK2 and increases the risk of breast cancer. Germline mutations/variants in CHEK2 were studied in high-risk African Americans from the Northern California site of the Breast Cancer Family Registry (n = 198) and Howard University Hospital (n = 62). High risk criteria for hereditary breast cancer included a family history of breast and/or ovarian cancer, bilateral breast cancer, or early-onset (age 40 years or less) breast cancer. BRCA1 and BRCA2 carriers were excluded. The entire CHEK2 coding and intron flanking regions were examined using denaturing high performance liquid chromatography and putative mutations/variants were delineated by DNA sequencing. Three novel silent mutations coding for the same amino acid were detected: 252A>G (Glu84); 660C>T (Tyr220), and 1497G>C (Leu497). Exonic splicing enhancer (ESE) analysis revealed that these mutations may negatively affect splicing; further studies are needed to support this possibility. One previously described missense mutation that does not affect function, 254C>T (P85L), was also detected as well as two intronic mutations. Multiplex ligation-dependent probe amplification (MLPA) did not identify any large rearrangements in CHEK2. Most importantly, these experiments detected two novel mutations: R180C and 1135delTC. Mutation R180C is not located in a functional domain but may affect protein folding and function as the mutation was deemed intolerant by the bioinformatic analysis tool Sort Intolerant From Tolerant (SIFT) that compares homologous sequences in different organisms. Mutation 1135delTC is protein truncating and located in the important kinase domain of CHEK2 in close proximity to the 1100delC mutation; therefore, 1135delTC is expected to be pathogenic. The yeast Rad53 gene is homologous to the human CHEK2 gene. The wild-type human CHEK2 gene has been demonstrated to complement the deleted yeast Rad53 gene. Therefore, yeast complementation allows evaluation of the effect of potentially pathogenic mutations. Site-directed mutagenesis of the human wild-type gene was used to generate plasmids with the 1100delC, 1135delTC, and R180C mutations. Introduction of the human wild-type CHEK2 gene into the Rad53 deleted yeast resulted in growth in selective media; whereas introduction of the latter three mutations yielded no growth in selective media and growth in control non-selective media. We conclude that the 1135delTC and R180C mutations are pathogenic because they are detrimental to the function of the protein. These findings are significant because 1135delTC is the only other protein-truncating mutation discovered to date in this gene. Screening for CHEK2 variations in the African American population should include the pathogenic R180C and 1135delTC mutations.

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P59-4: COP1, AN UBIQUITIN LIGASE OF p53, IS NEGATIVELY REGULATED BY 14-3-3 σ

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Constitutive photomorphogenic protein 1(COP1) was first identified as a negative regulator of photomorphogenic development in Arabidopsis. Arabidopsis COP1, a RING containing protein, targets HY5 transcription factor, activating light-inducible

genes, for proteasome-dependent degradation. In mammalian, COP1 overexpression was found in ovarian and breast cancers. Previous studies have shown that COP1, like MDM2, is a p53 E3 ligase and negatively regulates p53 stability. Recently, phosphorylation of COP1 by ATM disrupts COP1-mediated p53 stability. In our lab, we observed that 14-3-3 sigma (14-3-3 σ), a p53 inducible gene, blocks MDM2-mediated p53 ubiquitination and degradation, suggesting that 14-3-3 σ may negatively regulate COP1. Based on this, we hypothesize that 14-3-3 σ blocks COP1-mediated p53 stability.

First, COP1 and 14-3-3 σ were cotransfected in 293T cells. We found that increasing amounts of 14-3-3 σ cause down-regulation of COP1. To further examine whether 14-3-3 σ blocks COP1-mediated p53 ubiquitination, 14-3-3 σ , COP1, and p53 were cotransfected into 293T cells using His-beads pull down. We found that COP1-mediated p53 ubiquitination levels were reduced in the presence of 14-3-3 σ . We then investigate whether 14-3-3 σ affects COP1 turnover rate using cycloheximide, which blocks protein synthesis. Also, we found that amounts of COP1 were dramatically reduced in the presence of 14-3-3 σ . We used shRNA of 14-3-3 σ to knockdown endogenous 14-3-3 σ in U2OS cells and found that amounts of COP1 were stabilized when gene expression of 14-3-3 σ is inactivated. Then we found COP1 can interact with endogenous 14-3-3 σ using co-immunoprecipitation experiments. We will further characterize the domains that mediate the interaction between 14-3-3 σ and COP1.

To further investigate whether COP1 functions as an oncogene, we generated COP1 stable cell lines in U2OS cells, C16. We found C16 cells have increased proliferation rate relative to that of parental cells. However, 14-3-3 σ -infected C16 cells reduced proliferation rate compared with beta-gal infected C16 cells. We also found COP1 positively regulates cell migration using wound healing assay and transwell experiments. Because light-mediated Arabidopsis COP1 shuttles between nucleus and cytoplasm, we investigate whether mammalian COP1 is a shuttling protein in response to DNA damage signals. We found that COP1 was in the cytoplasm after DNA damage. We will further study COP1 tumorigenesis using nude mice to address its tumorigenicity.

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P59-5: RESULTS FROM A GENOME-WIDE LINKAGE SCAN USING A COLON-BREAST CANCER PHENOTYPE

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The goal of this project was to identify susceptibility genes for a joint colon/breast cancer phenotype. Colorectal cancer (CRC) is the third most commonly diagnosed cancer in Americans and is the second leading cause of cancer mortality. To identify susceptibility genes, the Colon Neoplasia Sibling Study (CNSS) conducted a comprehensive, genome-wide linkage scan. Clinical information (histopathology, size and number of polyps, and other primary cancers) was used in conjunction with age at onset and family history to classify families into five phenotypic subgroups (severe histopathology, oligopolypos, young, colon/breast, and multiple cancer) prior to analysis.

The Colon/Breast subgroup examines the strong clustering of breast and colon cancers noted in population-based studies. Cancer geneticists have speculated that there are likely to be "breast-colon" susceptibility genes. Families segregating Breast Cancer 1 (BRCA1), Breast Cancer 2 (BRCA2), and Checkpoint Kinase 2 (CHEK 2) mutations have an increased risk for colon cancer. But these relatively rare genes cannot explain the strong familial clustering of breast and colon cancers.

We identified 33 families in the CNSS with a case of breast cancer in a first-degree relative (FDR) and 172 families in which there was an individual affected with breast cancer in either an FDR or second-degree relative. Our analysis included only families with an FDR affected with breast cancer, as this allowed us to compare the allele sharing between breast and colon neoplasia cases. Families in this subgroup had at least two sibs who were affected with adenomatous polyps or colon cancer and a sib or mother with breast cancer.

We found linkage to BRCA2 (P value= 0.0070) in the breast/colon phenotypic subgroup and identified a second locus in the region of D21S1437 (P value= 0.0003) segregating with, but distinct from, BRCA2. We observed linkage to marker D17S1308, P value= 0.0086, (17p), in close proximity to the candidate gene Hypermethylated in Cancer 1 (HIC1 [MIM 603825]). HIC1 has not been previously associated with colon or breast cancer, but it is an attractive candidate gene. HIC1 is hypermethylated in cancer and is frequently silenced by epigenetic mechanisms [1]. In murine models, Chen et al. [2] demonstrated that disruption of HIC1 predisposes to gender-dependent tumors, with males more likely to develop epithelial cancers and females more likely to develop lipid and mesenchymal cancers, thus making HIC1 an attractive candidate for gender-dependent cancers in humans. We found no evidence for linkage to BRCA1 or CHEK 2.

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2. Chen, W.Y., et al., Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. *Nat. Genet.*, 2003. 33(2): p. 197-202.

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P59-6: URINARY 6-SULPHATOXYMELATONIN LEVELS AND RISK OF BREAST CANCER IN POSTMENOPAUSAL WOMEN: THE ORDET COHORT

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Several factors including light at night, age, a higher body mass index, and smoking, suppress melatonin production. Lower urinary melatonin levels have been associated with a higher risk of breast cancer in premenopausal women. The association between circulating melatonin levels and breast cancer risk in postmenopausal women remains unclear.

The Hormones and Diet in the Etiology of Breast Cancer Risk (ORDET) cohort was established in northern Italy between June 1987 and June 1992, when 10,786 healthy women ages 35 to 69 years, were enrolled. Women were excluded if they reported a bilateral ovariectomy, were currently breast feeding or pregnant, used oral contraceptives or hormone replacement therapy, were affected by metabolic diseases or reported a history of cancer. After an average of 17 years of follow-up, the ORDET data were linked with the local Lombardy Cancer Registry to identify breast cancer cases. Four control subjects were matched to each breast cancer case on age at recruitment (5 years); daylight saving period at recruitment; recruitment center; and date of recruitment. In a prospective case-control study nested within the ORDET, we measured the concentration of melatonin's major metabolite, 6-sulphatoxymelatonin (aMT6s), in the overnight urine of 178 postmenopausal women with incident breast cancer and 710 matched control subjects. Urinary aMT6s was assayed by the Hormone Research Laboratory, Etiological Epidemiology and Prevention Unit, at the National Cancer Institute Foundation (Milan, Italy), using the Bühlmann ELISA EK-M6S. Creatinine levels were also measured for each sample and aMT6s levels were normalized to the creatinine level of the sample.

Study participants were all postmenopausal with an age range between 41 and 70 years at urine collection. In logistic regression models, the multivariate relative risk [reported as the odds ratio (OR)] of invasive breast cancer for women in the highest quartile of urinary aMT6s compared to the lowest was 0.62 [95% confidence interval (CI) = 0.36 to 1.06; P_{trend} = 0.02]. After the exclusion of 29 women who reported smoking cigarettes at the time of urine collection, this association became even stronger (OR, 0.50, 95% CI, 0.27-0.94; P_{trend} = 0.003). Further, there was an increasingly stronger association between urinary aMT6s level and breast cancer risk after we excluded case patients who were diagnosed with invasive breast cancer within 2 or 4 years after their urine collection (OR for highest versus lowest quartile of urinary aMT6s concentration = 0.43, 95% CI = 0.21 to 0.87, P_{trend} = 0.005; and 0.36, 95% CI = 0.16 to 0.81, P_{trend} = 0.003, respectively).

These prospective data provide, to our knowledge for the first time, evidence for a significant, inverse association between higher melatonin levels, as measured in overnight morning urine, and breast cancer risk in postmenopausal women.

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P59-7: THE BREAST CANCER IN AFRICAN WOMEN STUDY: ASSESSMENT OF LIFESTYLE QUESTIONNAIRE AND PRELIMINARY GENOTYPE ANALYSIS

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Breast cancer is a disease with a complex etiology that consists of lifestyle and genetic components. When controlling for non-genetic factors, it is clear that African and African American women are more likely to develop aggressive tumors that result in higher mortality compared to Caucasian American women. This suggests that a comprehensive analysis of genetic and lifestyle factors that includes, minimally, these three demographic groups could provide valuable insights into the complex interactions that result in disease. To this end a pilot project, designed as a case-control study, was developed with collaborators in Ile-Ife, Nigeria and Nairobi, Kenya. The specific aims were (1) to

develop an internationally correlative, yet culturally relevant questionnaire with which to identify lifestyle risk factors in African women, and (2) to develop standard operation procedures for the collection, storage and shipping of blood, buccal cells, and urine for biomarker analysis.

A qualitative analysis of the questionnaires highlighted some fundamental changes that are necessary in order for this part of the study to be maximally effective. A number of questions were consistently unanswered. Possible reasons for this could include embarrassment at the subject matter for some questions and questionnaire fatigue for others. In some cases the questions were apparently confusing despite being administered by a trained interviewer. Also the individuals administering the questionnaire felt that the questionnaire was simply too long. There were also occasional issues of language in that the questionnaire is written in English and a number of individuals spoke only local dialects. The questionnaire is currently being revised to address a number of these points. Although the size of the study population is small, one interesting piece of demographic information was revealed that needs to be addressed in future studies which is that the socioeconomic status (SES) of the case and control populations was skewed in opposite directions. The cases were of higher SES than the controls. Specific breast cancer risk factors correlate with SES, making income a variable that must be controlled, in addition to age, when selecting for the control population. A comprehensive analysis of the utility of the questionnaire will be presented at this meeting.

In regards to the biological specimens collected for biomarker analysis, DNA was purified from the blood clots and the quality and quantity were adequate for genotyping. Genes involved in inflammation, detoxification and estrogen metabolism were selected for evaluation. Again, the number of samples is reflective of the pilot status of the project and too small for a stringent statistical analysis of the data but it is clear that the procedures that are in place in Ile-Ife and Nairobi, as well as Nashville, are adequate to support a study of this type. The work completed thus far on the survey and the initial preparation of samples for genotyping show promise and support moving towards the comprehensive goal of this work, which is to develop an infrastructure on which to build a larger study that includes African women and women of African descent located worldwide.

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P59-8: GENOMIC PROFILING OF SPORADIC AND BRCA-RELATED BREAST CANCER

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Germline mutations in the *BRCA1* or *BRCA2* genes confer increased breast cancer risk. Previous DOD-funded studies examined the frequency and breast cancer impact of *BRCA* mutations in the Icelandic population. Over 6,000 individuals participated in these studies who have since been followed up in a number of ways. Local founder mutations have been found in ~8% of all breast cancers in the population with one *BRCA2* mutation, 999del5 accounting for 6-7%. This *BRCA2* founder mutation has also been shown to be of importance in prostate cancer. In addition to germline abnormalities in the *BRCA* genes, we have demonstrated epigenetic silencing of the *BRCA1* gene in 10% of breast tumors. The objective of the present study was to examine the effects of *BRCA* dysfunction with respect to clinical relevance and prognosis with the specific aim of defining subgroups with potential molecular targets for *BRCA*-targeted treatment. Breast tumor subgroups are defined based on Comparative Genome Hybridization array (array CGH) copy number profiles and tissue microarrays (TMAs). The resulting subgroups are examined in terms of *BRCA* dysfunction and clinical relevance.

The study group included sporadic breast tumors and tumors derived from carriers of the Icelandic *BRCA2* 999del5 founder mutation. All tumors were analyzed for methylation of the *BRCA1* gene by methylation-specific PCR (MSP) and expression of *BRCA1* on tissue microarrays. To analyze copy number alteration profiles, we performed array CGH using high-resolution oligonucleotide microarrays (*NimbleGen Systems*). To examine clinical relevance, we obtained information on clinicopathological parameters including grade, nodal status, and time to relapse. In addition, we analyzed expression of several prognostic and/or phenotypic markers on TMA sections. Potential therapeutic targets are validated using immunohistochemistry or FISH on TMA sections. Partitioning cluster algorithms with average silhouette information revealed that the variability of genomic profiles are captured in three clusters, each one representing a distinguishing tumor subgroup as determined by the examined features. In pursuing the stated purpose, the focus concentrated on the subgroup found to be characterized by high prevalence of *BRCA* abnormalities, i.e., tumors with *BRCA2* mutation and/or absent *BRCA1* expression with or without *BRCA1* methylation. The association between *BRCA* abnormalities and their determined cluster was statistically significant without any identified confounding effects. The *BRCA* abnormal subgroup had the highest frequency of patients that experienced relapse within 5 years from surgery suggesting an aggressive phenotype.

Examination of the genomic segmentation profiles reveals a striking difference in the observed patterns in that the *BRCA* abnormal tumor subgroup tend to have long stretches of copy number alterations with deletions more prevalent suggesting defects in double-strand break (DSBs) repair. Indeed, *BRCA1* and *BRCA2* are known to be important in DSB repair by homologous recombination. These results support the hypothesis that the *BRCA* genes have a significant role in sporadic breast cancer. The importance of these findings lies in the potential benefits of *BRCA*-targeted therapy for a larger group of patients than the few breast cancer patients carrying a germline *BRCA* mutation.

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P59-9: GENETIC POLYMORPHISMS IN THE CATECHOL ESTROGEN METABOLISM PATHWAY AS MODIFIERS OF THE EFFECT OF HORMONE THERAPY IN BREAST CANCER RISK

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Combined hormone therapy (CHT) has been implicated as a risk factor for breast cancer but mechanisms remain unknown. Genetic variation in the catechol estrogen metabolism pathway may act to modify the effect of CHT and estrogen-alone hormone therapy (EHT) on breast cancer risk. In a population-based case-control study of breast cancer in women ages 65–79 in which CHT use was found to be associated with an increased risk of breast cancer, 891 cases and 878 controls were genotyped for functional single nucleotide polymorphisms (SNPs) in the *CYP1B1*, *COMT*, *GSTT1*, *GSTM1*, and *GSTP1* genes. Within single gene analyses, women who carried at least one copy of the A allele in the *GSTP1* gene (105 Ile; rs1695) had a 1.4-fold increased risk of breast cancer compared to those who were homozygous for the G allele (95% confidence interval (CI) 1.1–1.9); within the *CYP1B1**2 allele (119 Ser; rs1056827) women homozygous with the T allele were at 1.8 (95% CI:1.2–2.6) times the risk of those carrying at least one copy of the G allele; the relationship between these single genes and breast cancer is stronger in current, long-term CHT users than in never CHT users; no other single genes demonstrated significant associations nor did the other single genes have a significant interaction with CHT (ever use, current use, duration). In a multi-gene model limited to genes with single gene effects (*CYP1B1**2 and *GSTP1*), the risk of breast cancer for carriers of 1 high-risk genotype was 1.5 (95% CI: 1.01–2.3) times higher than women with 0 high risk genotypes, and the risk was 2.8 (95% CI:1.5–5.2) for women with 2 high-risk genotypes compared to those with 0. This association was heightened among current, long-term (60+ months) CHT users, (OR = 7.4 [95% CI 1.9–28.1]) for women with 1–2 high-risk genotypes compared to 0 genotypes, while in non-users of CHT, the association was attenuated (OR = 1.3 [95% CI 0.8–2.1]) for 1–2 versus 0 genotypes. These analyses were extended to histologic type and estrogen receptor/progesterone receptor subtypes. These results suggest that the risk of breast cancer among CHT users is modified by genetic variation in the estrogen metabolism pathway.

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P59-10: GENETIC POLYMORPHISMS IN THE SUPEROXIDE DISMUTASE-2 AND CATALASE GENES AND BREAST CANCER RISK: RESULTS FROM THE NASHVILLE BREAST HEALTH STUDY

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Reactive oxygen species (ROS) have long been thought to play a key role in breast carcinogenesis. The antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) are major antioxidant enzymes for ROS detoxification. Genetic constituent, which determines antioxidant enzyme expression and antioxidant synthesis, thus may be related to the risk of development of breast cancer. We evaluated the association of genetic polymorphisms in the *SOD2* and *CAT* genes with breast cancer risk in the Nashville Breast Health Study. Included in this study were 968 caucasian breast cancer cases and 863 caucasian controls who had completed an interview and donated a DNA sample to the study. Three functional or potentially functional polymorphisms were evaluated in the *SOD2* (rs1799725) and *CAT* (rs511895, rs1001179) genes by the TaqMan assay. We observed an increased risk of breast cancer among women with the *SOD2* rs1799725 TC genotype (odds ratio [OR] = 1.22, 95% confidence interval [CI], 0.98–1.52) compared to women with the TT genotype. The increased risk appears to be more evident among premenopausal women (OR = 1.34, 95% CI, 0.91–1.98) as compared to postmenopausal women (OR = 1.17, 95% CI, 0.89–1.55). The *CAT* gene polymorphisms rs511895 and rs1001179 were not associated with the risk of breast

cancer. Results of this study provide some evidence that genetic polymorphisms in the *SOD2* gene may be associated with the risk of breast cancer.

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P59-11: ASSOCIATION STUDIES OF REGIONAL GENOME SCAN TO IDENTIFY BREAST CANCER SUSCEPTIBILITY GENES

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Genetic susceptibility is believed to play an important role in the development of most cancers including breast cancer. A cost-efficient approach to search for breast cancer susceptibility genes is to investigate chromosome regions that are known to be linked to breast cancer. The loss of heterozygosity (LOH), particularly in the 7q31.2 and 16q24.3 chromosome regions, is the most common type of somatic alteration found in breast cancers. LOH has been widely reported to correspond to the loss of the second allele of a tumor suppressor gene. We hypothesize that the 7q31.2 and 16q24.3 chromosome regions may harbor breast cancer susceptibility genes and germ-line sequence variants in these genes may increase the risk of breast cancer. To test this hypothesis, we are conducting a multiple-phase study using resources of the Shanghai Breast Cancer Study (SBCS) and the Nashville Breast Health Study (NBHS). Thus far, we have completed genotyping of dense SNPs in the 7q31.2 and 16q24.3 regions for cases and controls of the SBCS. We are analyzing the data to identify genes associated with breast cancer risk and will re-sequence functional regions (promoter, exons, exon-intron boundaries) of these candidate genes in 25 cases and 25 controls from the SBCS. We will evaluate the sequence variants identified in the candidate gene with breast cancer risk in an independent set of 800 cases and 800 controls from the SBCS. Finally, we will conduct a confirmation test on promising polymorphisms among 800 cases and 800 controls participated in the NBHS. The proposed study, through its use of robust analytic and statistical methods and existing unique resources, should provide credible results allowing us to conclude whether the 7q31.2 and 16q24.3 regions harbor breast cancer susceptibility genes and whether sequence variants in these genes increase the risk of breast cancer.

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P59-12: CHEK2 1100delC VARIANT AND BRCA1 AND BRCA2 NEGATIVE FAMILIAL BREAST CANCER – A FAMILY-BASED GENETIC ASSOCIATION STUDY

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Background: The human checkpoint kinase 2 (CHEK2), the human ortholog of yeast Cds1 and Rad53, encodes a cell-cycle checkpoint kinase that plays a role in DNA repair processes involving BRCA1 and p53 and is thus a candidate gene for familial breast cancer and Li-Fraumeni Syndromes (LFS)¹. While several germline missense mutations in the *CHEK2* gene have been reported in LFS and LFS-like families one particular deletion mutation (1100delC) in the *CHEK2* gene, which clearly abrogates the kinase activity of the encoded protein, has recently been shown to increase the risk of breast cancer in high-risk families negative for BRCA1/2 mutations as compared to population controls.

Technical Objective: Using a family-based design, we examined the prevalence and penetrance of *CHEK2**1100delC variant and its association with breast cancer among BRCA1/2 negative families from five North American centers participating in the NCI's Breast Cancer Family Registry (BCFR) for whom genomic DNA and questionnaire and family data have already been collected.

Method: We employed a nuclear family-based design (that avoids population stratification bias) which can use families with arbitrary structure (i.e., varied numbers of affected/unaffected and typed/untyped members) to examine the *CHEK2**1100delC-breast cancer association. The *CHEK2* single base deletion (1100delC) variant was genotyped by a PCR (to specifically amplify *CHEK2* exon 10 on chromosome 22) followed by single nucleotide extension on the amplified product using fluorescence polarization to detect incorporated nucleotide. All identified mutations are confirmed by direct sequencing.

Results: The formal statistical analyses of the above mentioned objectives are under way. However, preliminary analyses suggest that the 0.64% of the 3,883 individuals genotyped carry the *CHEK2**1100delC mutation. The prevalence was nearly 2% among women affected with breast cancer and 0.2% among women unaffected with breast cancer in the same families. A formal modified segregation analyses to estimate the penetrance and relative risk of *CHEK2**1100delC mutation in relation to breast cancer are being conducted now and the findings will be presented in the meeting.

Conclusion: Given the biological relevance of CHEK2 in breast carcinogenesis and loss of CHEK2 function due to the 1100delC variant and there is a compelling need for independent confirmation and characterization of the recently observed association between the *CHEK2**1100delC variant and breast cancer. This family-based study accomplished that by employing robust analytic methods using existing unique resources. Analyses of a large number of carefully selected and well-characterized families as well as the established collaborative set-ups are major strengths of the study. The estimated absolute (penetrance) and relative risks for the *CHEK2**1100delC mutation for breast cancer reported from this study will be of significant clinical importance.

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P59-13: ROLE OF CYP1B1 IN PAH-DNA ADDUCT FORMATION AND BREAST CANCER RISK

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This study investigated the hypothesis that widespread exposures to ubiquitous pollutants, namely, polycyclic aromatic hydrocarbons (PAHs), are related to increased breast cancer risk. PAHs are products of incomplete burning of organic matter and are present in cigarette smoke, ambient air, drinking water, and diet. PAHs require metabolic transformation to bind to DNA, causing DNA adducts, which can lead to mutations and are thought to be an important pre-cancer marker. In breast tissue, PAHs appear to be metabolized to their cancer-causing form primarily by the cytochrome P450 enzyme CYP1B1. The level of the CYP1B1 enzyme depends on both genetic and environmental factors and high CYP1B1 levels are suspected to result in increased formation of PAH-DNA adducts, thus increasing breast cancer risk. We have previously reported that expression of the *CYP1B1* gene in non-tumor breast tissue is markedly higher in breast cancer cases than in controls. Here we report on our investigation of underlying molecular mechanisms of the relationship between PAH exposure and breast cancer risk.

Using a clinic-based case-control design, breast tissue was obtained from female patients undergoing either mastectomy (cases) or reduction mammoplasty (controls) in Porto Alegre, Brazil. We chose this location because high levels of PAH and other chemical exposures are found there, air pollution controls in the area are limited and the local diet includes frequent barbecued meat consumption.

To characterize molecular level inter-individual variation in PAH metabolism, normal breast tissue was collected from 54 cases and 15 controls. (The number of control participants was much lower than anticipated because of economic factors and changed beauty perception.) PAH exposure and potential confounding factor data were collected from consenting participants via medical chart review and an interviewer-administered questionnaire. Breast tissue was preserved in RNALater buffer and subsequently shipped to LBNL for analysis of *CYP1B1* gene expression polymorphism and PAH-DNA adducts. Evaluation of PAH exposure and potential confounders is ongoing and will not be reported here.

CYP1B1 expression level in the breast specimens was measured using real-time RT-PCR and compared to a *CYP1B1* quantitation standard. *CYP1B1* transcript levels varied from the detection limit of 0.05 to 73 fg/500 ng RNA (similar to the range we had observed previously in women from the Bay Area) with a mean of 21.49 ± 20.37 fg/500 ng RNA for cases and 13.44 ± 14.75 fg/500 ng RNA for controls. The *CYP1B1* genotypes at two polymorphic sites (codon 432 and codon 453) were analyzed by PCR/RFLP. The allele frequencies determined in our small sample set resemble those seen by others in populations of European descent. PAH adduct levels in DNA were determined by the nuclease P1-enhanced version of the ³²P-postlabelling assay. PAH-DNA adducts levels ranged between 2 to 107/10⁹ nucleotides with a mean value of 33/10⁹ nucleotides in both cases and controls.

Our analysis to date has not found any obvious differences in the mean values in any of the parameters we have measured. We will undertake a more comprehensive analysis once the questionnaire responses are evaluated.

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P59-14: MITOCHONDRIAL INFLUENCE ON BREAST CANCER METASTASIS SUSCEPTIBILITY

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Mitochondria are important cellular organelles with critical functions related to energy production, thermogenesis, programmed cell death, growth, and cell signaling. Not surprisingly, mitochondria have also been implicated to play a significant role in the

etiology of a variety of diseases, including cancer. More recently, it has been noted that certain human mitochondrial DNA haplotypes appear to increase the risk for certain types of cancer and tumor growth in mice. Interestingly, it is also known that genetic background influences metastatic mammary tumor formation in mice although these studies have not examined the potential for mitochondrial–nuclear interaction as a determinant for metastatic susceptibility. Interestingly, based upon BLAST sequence-comparisons (<http://www.ncbi.nlm.nih.gov>), differences appear to exist between metastasis-susceptible and resistant mice (e.g., C57BL/6J versus NZB/B1NJ mice, respectively). Herein it is hypothesized that mitochondrial haplotype is an important factor in influencing breast cancer metastasis susceptibility. To test this hypothesis, a molecular genetic approach is proposed that will investigate the role of mitochondrial genetic background on breast cancer metastasis. To determine whether mtDNA from metastatic-prone and resistant mouse strains influences breast cancer metastasis, mice known to be susceptible (C57BL/6J) or resistant (NZB/B1NJ) to metastasis in a metastatic mammary tumor model (FVB/N-TgN(MMTVPyMT) transgenic mouse) will be used in “nuclear exchange” experiments to generate mice with a C57BL/6J mtDNA haplotype and FVB/N-TgN nuclear genome and a mouse with a NZB/B1NJ mtDNA and FVB/N-TgN nuclear genome. These mice will then be assessed for metastatic tumor formation. These studies will determine whether mitochondrial genetic background influences individual metastasis susceptibility. Consequently, if the tested hypothesis proves correct, mitochondrial genetic background could be determined in individuals considered to be at risk for breast cancer metastasis to determine the appropriate therapeutic approach.

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P59-15: ABSTRACT WITHDRAWN

P59-16: DISRUPTION OF PERIPHERAL CIRCADIAN RHYTHM GENES INITIATE BREAST TUMORIGENESIS

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Exposure to artificial light correlates with higher incidence of breast cancer. Shift workers, whose day/night rhythms are altered by their odd hours, appear more prone to develop breast cancer. In response to natural light, a master clock in our brain regulates molecular clocks in cells of the peripheral tissues, triggering clock-regulated genes that govern fundamental cellular functions. Critical clock genes—the period genes *PER1*, *PER2*, and *PER3*—were found to be downregulated in breast cancer. It is currently unknown whether disruption of the peripheral clock in human breast epithelial cells leads to transformation. By using a modified serum shock protocol, we entrained human untransformed breast epithelial cells in vitro and found that a few key clock genes, including the *PER* genes are transcribed in a rhythmic fashion in untransformed but not in transformed breast epithelial cells. We tested whether disruption of one of the key clock genes, *PER2*, can induce breast epithelial transformation in vitro. We found that stable knock down of *PER2* in a human untransformed breast epithelial cells by RNA interference leads to aberrant three-dimensional morphological phenotypes similar to the phenotypes of early breast cancer. Thus factors that can lead to an aberrant peripheral circadian rhythm might be capable of initiating breast tumorigenesis.

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TRANSCRIPTION, TRANSLATION, AND MODIFICATION

Poster Session P60

P60-1: THE TRANSCRIPTION FACTOR C/EBP β 2 MEDIATES ErbB INDEPENDENCE AND TRASTUZUMAB RESISTANCE

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Although there is a significant improvement in relapse-free survival when patients with ErbB2 (HER2/neu) overexpressing breast cancer receive trastuzumab in addition to adjuvant chemotherapy, intrinsic and acquired resistance to trastuzumab continue to be a clinical concern. Recent studies indicate overexpression of ErbB1 (EGFR) may confer resistance to trastuzumab. This is supported by data demonstrating tykerb, which inhibits both ErbB1 and ErbB2, synergizes with trastuzumab and has clinical activity in trastuzumab-resistant tumors. The transcription factor C/EBP β 2, however, confers EGF independence in MCF10A cells that is independent of ErbB signaling. C/EBP β 2 is expressed at high levels in 70% of invasive mammary carcinomas, but is not present in normal mammary tissue. C/EBP β 2 is downstream of multiple signaling pathways, including those that activate the ERK and RSK kinases. ERK and RSK kinases are often activated by the ErbB family of receptor tyrosine kinases in breast cancer via the Shc- and/or Grb2-activated Ras-Raf-MAPK pathways and phosphatidylinositol-3-kinase (PI-3 K) pathways. It has been demonstrated that CEBP β is necessary for Ras transformation in multiple cell types. Thus, it is probable that C/EBP β 2 is a downstream effector of ErbB signaling in mammary epithelial cells. As C/EBP β 2 is often overexpressed in breast cancer and may be an important downstream effector of ErbB signaling, we sought to determine the mechanism of C/EBP β 2 mediated EGF independence in MCF10A cells. We first characterized C/EBP β 2 regulation of gene expression in MCF10A cells using genomic profiling. Pathway analysis confirmed known contributors to ErbB independence are not upregulated in MCF10A cells overexpressing C/EBP β 2. However, three members of the interleukin 1 family, known to be overexpressed in breast cancer, are upregulated 30-fold or more. They are IL1R2, IL1B, and ST2 (IL1RL1). Chromatin immunoprecipitation studies showed that IL1B is directly regulated by C/EBP β 2. ELISA and immunoblot analysis demonstrated the presence of active and proform of IL1B protein upon C/EBP β 2 overexpression. Surprisingly, immunofluorescent staining and cell fractionation studies revealed the proform of IL1B is localized to the nucleus. Neutralizing antibodies against IL1B and its receptors were used to determine the role of secreted IL1B in EGF independent growth. No effect was observed indicating nuclear IL1B may play an important role in our system. We are currently using shRNA mediated knock-down of IL1B to ascertain the importance of nuclear IL1B in EGF independence in MCF10A cells. Given that MCF10A cells overexpressing C/EBP β 2 are no longer dependent on ErbB signaling for survival, we sought to investigate whether aberrant C/EBP β 2 expression could contribute to the resistance of some breast cancers to ErbB targeted therapies, such as trastuzumab. C/EBP β 2 was retrovirally introduced into ErbB2 overexpressing cell lines sensitive to trastuzumab. These cells were then assayed for Herceptin sensitivity and it was found that C/EBP β 2 confers resistance to trastuzumab. By uncovering and understanding the alternative signaling pathway(s) present in trastuzumab-resistant tumors, this research may uncover new targets for therapy of trastuzumab-resistant breast cancer.

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P60-2: ROLE OF ANT ISOFORM EXPRESSION IN CONTROLLING ENERGETICS, PROLIFERATION, AND SURVIVAL OF BREAST CANCER CELLS

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In agreement with the Warburg hypothesis, the energy demands of breast cancer cells are satisfied largely through aerobic glycolysis, whereas normal cells rely on oxidative phosphorylation in the mitochondria. Understanding the mechanistic basis of this metabolic switch is fueling exciting new strategies using glycolytic inhibitors as anti-cancer agents; but since mitochondria are central to both energy production and apoptosis, mitochondrial proteins are also likely to play key roles in regulating energy production and apoptosis in breast cancer cells. The adenine nucleotide translocase (ANT) normally functions to co-transport ATP from the mitochondria to the cytosol and ADP in the reverse direction. ANT2 has several features distinct from the well-studied ANT1 isoform. Silencing of ANT2 results in chemosensitization of HeLa cells. Ectopic expression of ANT1, but not of ANT2, induces apoptosis in some cell lines. ANT2, originally identified as a nuclear-encoded immediate early gene, is expressed in highly proliferating cells, including breast tumors. Examination of the putative promoter regions of the ANT isoforms for transcription factor binding sites present in ANT2, but not in ANT1, revealed that the highest scoring candidate was c-Myc. Overexpression or activation of c-Myc is required for proliferation of many breast cancer cells, including estrogen (E)-induced proliferation of hormone-responsive MCF-7 cells. Recognition of these features has prompted us to explore the innovative concept that ANT2 is a novel target of E and Myc, with transport activity that differs from ANT1, which either causes a shift in ATP synthesis flux from mitochondrial to glycolytic pathways, or facilitates breast cancer cell survival/proliferation under conditions of aerobic glycolysis. An ideal model for testing these ideas is MCF-7 cells in which E, but not tamoxifen (Tam), induces c-Myc expression, promotes proliferation, and enhances glycolysis (4,5).

In the first phase of this project we are determining whether ANT2 is a novel E/c-Myc target gene required for E-induced survival/proliferation of breast cancer cells. We hypothesize ANT2, but not ANT1, mRNA levels will be induced upon E treatment and repressed upon Tam treatment of MCF-7 cells, in parallel with the well-established c-Myc regulation by E and Tam. If E-induced ANT2 expression requires c-Myc, then knockdown of c-Myc in MCF-7 cells should block E-mediated induction of ANT2 mRNA. The effect of ANT2 (versus ANT1) knockdown on E-induced MCF-7 cell proliferation, cell survival, and colony formation in soft agar is being determined. In the second phase of this project the impact of ANT isoform knockdown on bioenergetics and ANT transport activity of E-stimulated MCF-7 cells will be determined.

Two-thirds of breast tumors are E-responsive, but some tumors develop resistance to anti-estrogen therapies. Therefore it is critical to identify new therapeutic targets for breast cancer treatment. ANT2 is proposed to be an estrogen-induced gene that is important for energy production in breast tumor cells, allowing them to survive and proliferate. Inhibiting ANT2 activity or destroying its expression using new siRNA technologies may provide a novel means of killing breast tumor cells.

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P60-3: TRANSCRIPTIONAL REGULATION OF CREB-BINDING PROTEIN (CBP)

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Background: CREB-binding protein (CBP) is a tightly regulated transcription factor that is critical for regulating proliferation, differentiation, and apoptosis and is at the intersection of a plethora of signaling pathways. Suppression of CBP results in high-grade mammary atypia in CBP (+/-) transgenic mice and loss of CBP has been seen in some early-stage breast neoplasms. While it is well established that CBP is a co-activator of retinoid-signaling, the transcriptional regulation of CBP itself is uncharacterized.

Methods: Initial characterization of the CBP promoter was performed using promoter bashing, reporter plasmid transfections, and mutagenesis techniques. We have also identified, using bisulfite-sequencing, a region of the putative CBP promoter, that is methylated in cancer cells but not in normal cells, proximal to a consensus retinoic acid response element (RARE).

Results: We observe that the CBP RARE promoter sequence (1) is hypermethylated in breast cancer cell lines but not in normal mammary epithelial cells and (2) methylation corresponds inversely with CBP mRNA levels. In breast cancer cells hypermethylated at this sequence proximal to the RARE, treatment with 15 μ M 5-Aza-deoxycytidine agent causes an increase in CBP mRNA expression. This suggests that an epigenetic regulation of the CBP promoter is exerting some control over CBP transcription.

Conclusions: Our studies on CBP will facilitate our understanding of the transcriptional regulation of this key player in mammary gland homeostasis that is currently not studied widely. We also hope to develop this gene as a biomarker to predict the risk of a woman to develop breast cancer. Such a biomarker could also be used to monitor the success of chemoprevention agents. This translational project could have an important impact on basic breast cancer research, as well as patient care.

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P60-4: DIRECT VISUALIZATION OF ESTROGEN RECEPTOR-MEDIATED TRANSCRIPTION IN LIVING CELLS

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Estrogen stimulates proliferation of breast cancer cells whereas antagonists oppose their action. To study the molecular mechanisms of ligand-dependent regulation of transcription, we generated a cell line derived from a parent cell line containing an integrated tandem array of a mouse mammary tumor virus/Harvey viral ras (MMTV/v-Har-ras) reporter and a vector for cherry red fluorescence-estrogen receptor (ER)pbox mutant that recognizes glucocorticoid response elements (GREs). We observed ERpbox binding to the tandem array in an estradiol (E2)-dependent manner. We also observed concurrent transcription by RNA fluorescent in situ hybridization (FISH). RNA transcription correlated with ERpbox signal on the tandem array until steady state levels were reached. Chromatin immunoprecipitation (ChIP) assays showed recruitment of ERpbox to MMTV promoter and endogenous serum- and glucocorticoid-regulated protein kinase (Sgk), promoter. These studies in live cells demonstrate ERpbox binding to the MMTV promoter and transcription in an E2-dependent manner. Moreover, they demonstrate that ER pbox fusion protein also can bind to the GRE of an endogenous target gene. Live cell imaging using ERpbox and the MMTV tandem array, in combination with ChIP and RNA FISH, are powerful techniques to directly visualize the mechanisms of transcriptional regulation by E2 and selective estrogen

receptor modulators (SERMs) used for treatment of breast cancer. This cell line also may be a rapid and useful tool for drug screening of novel SERMs. Additionally, these studies raise the possibility that minor amino acid substitutions in the DNA-binding domain of nuclear hormone receptors can be engineered to alter the entire profile of cellular genes that respond to a particular hormone.

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P60-5: REPRESSION-INDUCED GENE SILENCING IN MAMMALIAN CELLS

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Gene silencing plays an important role in breast cancer development. Critical genes that are frequently silenced in human breast tumors include BRCA1, MLH1, E-cadherin (E-CAD), and estrogen receptor alpha (ER). A common factor for these genes is that each is repressible by environmental factors, which led us to propose that transcriptional repression acts as a trigger for gene silencing. A corollary of this hypothesis is that selection, which is a common fact in cancer, helps propel the silencing process. To test this hypothesis, the tetracycline (tet)-off system was used. This system has two components. The first is a promoter construct that contains tet binding elements and a minimal CMV promoter to drive gene expression, and the second is a construct expressing an activator protein that binds the tet-CMV promoter to induce promoter expression. Function of the activator protein is inhibited by adding the tet analog doxycycline (Dox) to the culture medium. Thus, promoter expression is normally at a high level and is repressible by adding Dox to the cell culture medium. The tet-CMV promoter was fused to the selectable HPRT cDNA and stably transfected into mouse cells expressing the activator protein. Due to the high level of HPRT expression, these cells were sensitive to the purine analog 6-thioguanine (TG). The only known mechanism for TG resistance is complete loss of HPRT activity/expression and few or no (< 10-5) TG-resistant clones were observed when several cell lines containing the tet-CMV-HPRT construct were plated in the presence of TG. Quantitative real-time PCR (qRT-PCR) demonstrated that exposure of the cells to Dox for 2–7 days reduced HPRT expression to low, but detectable levels. Consistent with this observation, the Dox-treated mass cultures remained somewhat sensitive to TG even in the continued presence of Dox. However, TG-resistant clones were identified at frequencies ranging from 10–2 to 10–4 after Dox treatment for 5–21 days followed by TG selection in the absence of Dox. The frequency of TG-resistant clones increased as a function of the duration of Dox exposure. These results suggested that transient repression of gene expression induced gene silencing in a small fraction of cells. Silencing, as opposed to mutation, was confirmed by demonstrating restoration of HPRT expression after treatment of the TG-resistant cells with DNA methylation and/or histone deacetylase inhibitors. A bisulfite sequence analysis confirmed that construct DNA methylation contributes to epigenetic loss of HPRT expression. Building upon these results, experiments are currently under way to determine if environmental repression of a breast cancer-relevant gene promoter, BRCA1, induces gene silencing. BRCA1 expression is repressed by hypoxia, which is common during tumor development. The BRCA1 promoter was linked to the HPRT cDNA and transfected into the parental mouse cells used for the above experiments and transfectants isolated in which HPRT expression is repressed when the cells are placed under hypoxic conditions. The protocol described above for Dox repression will be reproduced to determine if hypoxia-mediated repression can induce BRCA1 promoter silencing. A successful outcome will demonstrate a mechanism for the induction of gene silencing in breast cancer.

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P60-6: HISTONE MODIFICATIONS DOMINATE TRANSCRIPTION FACTOR EFFECTS IN REGULATING pS2 GENE EXPRESSION

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Background: The pS2 gene is expressed in breast tumors and involved in tumor cell migration. It binds estrogen receptor alpha (ER) transcription factor and is induced by estrogen in cell culture models of ER+ breast cancer. Chromatin structure also plays an essential role in regulating gene expression. Nucleosome assembly and histone modifications act in concert with other factors such as DNA sequence and transcription factor binding to regulate gene expression. ER+ tumors often progress to estrogen independence and develop resistance to treatment with tamoxifen. We expect that the molecular basis of estrogen independence and tamoxifen resistance to be multifactorial and include changes in the regulation of estrogen target genes. We studied the effect of an altered chromatin environment on the activity of the estrogen-responsive pS2 promoter.

Methodologies: We created stable cell lines with the pS2 promoter at two different chromatin sites in the same cell. We used the Flp-In system that allows for site-specific recombination to make these lines with 1.6 kb of the pS2 promoter regulating a firefly luciferase reporter whereas the native pS2 gene remained unaltered.

Results: Transcription from the native promoter site was hardly detectable in vehicle-treated cells and strongly induced by estradiol (E2) treatment. However, transcription at the Flp site, measured as firefly mRNA levels, was high in vehicle-treated cells and only weakly induced by E2 treatment. The high mRNA levels in the absence of E2 suggests that the Flp site is a highly transcriptionally active site. ChIP analyses around the estrogen-responsive element (ERE) revealed higher histone H3 and H4 acetylation (acH3 and acH4), as well as trimethylated H3K4 (H3K4me3) at the Flp site compared to the native site in vehicle-treated cells. However, E2 treatment did not change any of the histone modifications at either site. ER recruitment to both promoters occurred after E2 treatment but was higher at the Flp site. Trichostatin A (TSA), a histone deacetylase inhibitor, treatment resulted in an increase in acH4 but only modest increases in acH3, ER binding, and basal transcription at the native pS2 site. TSA treatment also caused an increase in E2-induced ER recruitment to both sites but failed to increase transcription at the Flp site. There was no difference in micrococcal nuclease sensitivity of the pS2 promoter in the Flp site compared to the native site. These data suggest that local histone acetylation and histone H3K4 trimethylation can regulate the basal pS2 transcription levels at both chromatin sites. Histone acetylation and histone H3K4 trimethylation regulate E2-dependent ER accessibility to the pS2 promoter at both sites but do not alter the maximum level of pS2 expression induced by E2. There appears to be a maximum level of gene expression from the pS2 promoter beyond which neither histone acetylation, H3K4 trimethylation, nor ER recruitment can substantially increase transcription.

Conclusions/Impact on Breast Cancer Research: We conclude that the presence of histone modifications overrides the function of the ER transcription factor in regulating pS2 gene expression. This mechanism for the loss of estrogen-regulated gene expression could contribute to the pathway for development of estrogen-independent breast tumors.

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P60-7: THE XIST NONCODING RNA FUNCTIONS INDEPENDENTLY OF BRCA1 IN X-INACTIVATION

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Mutations in breast cancer associated gene-1 (BRCA1) are associated with hereditary breast and ovarian cancers. BRCA1 encodes a ubiquitin ligase that acts in checkpoint and DNA damage repair pathways to ensure genome integrity. In addition, BRCA1 plays a role in meiotic XY inactivation. BRCA1 has also been implicated in regulation of somatic cell X-inactivation, leading to the suggestion that the loss of BRCA1 in female cells may lead to Xi perturbation and destabilization of its silenced state. This finding impacted both the breast cancer and X-inactivation research communities.

In female mammals, one X chromosome is silenced in each cell to achieve equivalent X-linked gene dosage between XX females and XY males. X-inactivation occurs early in mammalian embryogenesis, and in the mouse, the Xist gene is essential for the initiation of X-chromosome silencing. When X-inactivation is initiated, Xist RNA spreads from its site of transcription to coat the X chromosome, and this cis-spread correlates with the initial transcriptional silencing of the Xi. During the maintenance phase of X-inactivation, an accumulation of XIST RNA coats the Xi in somatic cells. Deletion of the XIST/Xist gene in somatic cells does not result in complete reactivation of the Xi. Instead, there is stochastic and infrequent gene reactivation, indicating Xist acts with other factors to maintain silencing of the Xi.

Ganesan et al. (*Cell*, 111:393-405, 2002) presented several lines of evidence implicating BRCA1 in regulation of X-inactivation. First, BRCA1 was enriched with the XIST RNA-coated chromatin of the Xi in female cells. Second, localized XIST RNA was not detected in BRCA1-mutant tumors and cell lines. Third, when wild-type BRCA1 was used to reconstitute a BRCA1-deficient cell line, XIST RNA was detected in a pattern consistent with its association with an Xi chromosome. Fourth, XIST RNA was no longer detected on the Xi when BRCA1 was depleted by RNA interference. Fifth, while XIST RNA was not detected in BRCA1-/- tumors, it was correctly localized in BRCA1-positive sporadic breast cancer samples. Finally, knockdown of Brca1 activated expression of an Xi-linked GFP, consistent with a role in maintaining X inactivation. Together, these results suggested that BRCA1 contributes to the stable silencing of X-linked genes by regulating the localization of XIST RNA. These findings were significant because BRCA1 was the first factor implicated in playing a role in XIST RNA localization and because they provided a possible mechanism by which loss of BRCA1 could contribute to cancer progression.

However, our studies to extend the findings of Ganesan et al., indicate BRCA1 does not regulate XIST RNA. We report that BRCA1 did not colocalize with XIST RNA in any cell type assayed. Reconstitution of BRCA1 in BRCA1-mutant cell lines did not affect XIST RNA distribution. Depletion of BRCA1 in wild-type cells did not alter XIST RNA localization. In mouse tumor cells mutant for Brca1, Xist RNA exhibited a normal distribution in 11/14 primary tumor lines. A human breast cancer cell line expressing mutant BRCA1 also showed normal XIST RNA distribution in the majority

of cells. Finally, dosage compensation in mouse *Brcal* mutant embryos and adult mammary tissues was normal. In combination, these results do not support a role for *BRCA1* in regulation of *XIST* RNA localization or its function in X-inactivation.

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P60-8: ANALYSIS OF EZH2 FUNCTION AND TARGET GENE SILENCING IN BREAST CANCER CELLS

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EZH2 is the catalytic subunit of polycomb repressive complex 2 (PRC2), which is a highly conserved chromatin-modifying enzyme complex. PRC2 methylates histone H3 on lysine 27, which is a repressive chromatin mark that contributes to gene silencing. Recently, EZH2 has been linked to aggressive forms of breast cancer as well as other cancers. Analyses of patient samples show that abnormally elevated EZH2 levels correlate significantly with invasiveness and increased proliferation rates of breast carcinomas (Kleer et al. 2003; *PNAS* 100:11606). In addition, EZH2 overexpression in breast epithelial cells causes anchorage-independent growth and increased cell invasiveness in vitro and injection of EZH2 overexpressing cells into mammary tissue causes tumor production in mice (Kleer et al. 2003; *PNAS* 100:11606; Cha et al. 2005; *Science* 310:306). EZH2 has also been functionally linked to CpG DNA methylation (Vire et al. 2006; *Nature* 439:871). Tumor suppressor genes are frequently hypermethylated on CpG and silenced during breast cancer progression. To address mechanisms by which EZH2 overabundance could alter cellular phenotypes, it is important to identify and study regulatory regions of target genes that undergo EZH2-dependent chromatin changes in mammary cells. After screening EZH2 target genes identified in non-mammary cell types (Kirmizis et al. 2004; *Genes Dev* 18:1592; Squazzo et al. 2006; *Genome Res* 16:890), we found that the transcription factor MYT1 and cyclin D2 (*CCND2*) are direct EZH2 target genes in breast cancer cells. Depletion of EZH2 by RNAi in SKBR3 cells derepresses MYT1 by about 20-fold and *CCND2* by about 5-fold. These EZH2 RNAi-treated cells also show decreased global levels of trimethylation on histone H3-K27. Chromatin immunoprecipitation (ChIP) assays show that EZH2 binds directly to the promoter and upstream regulatory regions of MYT1 and *CCND2*. We mapped EZH2 distribution in *CCND2* upstream DNA to a region from -1.3 kb to -3.2 kb, which defines a putative EZH2 response element. In agreement with a link between EZH2 and DNA methylation, we found that EZH2 knockdown reduces levels of CpG methylation in this *CCND2* region. PRC2 often functions together with other polycomb proteins in target gene silencing. In the *Drosophila* system, a DNA-binding protein called PHO helps recruit PRC2 to target genes, and histone methylation by PRC2 is thought to help recruit PRC1. We used ChIPs to address if the human homologs of these factors cooperate with PRC2 in SKBR3 cells. We find that YY1, the human homolog of PHO, and BMI-1, a subunit of human PRC1, are both recruited to the *CCND2* upstream region where they tightly colocalize with EZH2. These results suggest that conserved polycomb components cooperate in chromatin silencing at the *CCND2* locus in breast cancer cells. In the short term, we plan to perform functional assays to delimit the EZH2 response element(s) in this region. In the long term, we wish to fuse EZH2 response elements to reporter genes to identify EZH2 chemical inhibitors using cell-based transcription readout screens. The identification of EZH2 inhibitors may provide new drug leads for development of anti-cancer therapeutics.

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P60-9: GENE EXPRESSION IN AFRICAN AMERICAN AND CAUCASIAN AMERICAN BREAST CANCERS

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African American (AA) women have a higher breast cancer mortality than Caucasian American (CA) women. Many studies have demonstrated that this racial disparity in survival corresponds to a greater prevalence in AA women of negative prognostic factors, such as estrogen receptor-negative, progesterone receptor-negative (ER-/PR-) tumors, and early onset disease. Through an ongoing collaboration between Howard University and Johns Hopkins University, a significantly higher incidence of hypermethylation of five genes was observed in AA compared to CA women with ER- and PR- tumors diagnosed at age less than 50. Affymetrix gene expression microarrays were used to further investigate the molecular basis for the racial disparity in the ER-/PR-, age less than 50 subgroup. Epithelial invasive ductal carcinoma (IDC) cells were laser capture microdissected from frozen tissue. Normal epithelial cells from mammaplasty samples were isolated with epithelial-specific antibody-coated beads. Amplified, biotin-labeled anti-sense RNA was hybridized to Affymetrix U133 Plus 2.0 expression microarrays. AA and CA tumors exhibited both unique gene expression profiles and

quantitatively different levels of gene expression. Samples that are "unique" to one race are most likely those occurring at a higher or lower frequency in the ER-PR-, age less than 50 subgroup of that race. AA and CA samples used for the microarrays were not matched with respect to HER2 immunohistochemical expression. Overexpressed or underexpressed genes in AA carcinomas compared to CA tumors were investigated further using real-time quantitative reverse-transcriptase polymerase chain reaction (Q-RT-PCR); data were normalized to glyceraldehyde 3-phosphate from the same sample (Ct of the test gene minus Ct of glyceraldehyde 3-phosphate). Data from 13 frozen AA IDC, 12 frozen CA IDC, 13 AA normal breast organoids, and 12 CA normal breast organoids were compared. Collagen 5A1 (*COL5A1*) was significantly overexpressed in AA IDC compared to CA IDC (0.024); in AA IDC compared to AA normal breast (p = 0.0066); and in CA IDC compared to CA normal breast (0.0351) using Mann-Whitney analysis. *COL5A1* is involved in cell communication and indirectly linked to mitogen-activated protein kinase (MAPK) signaling. Cyclin A2 was significantly overexpressed in AA IDC compared to AA normal breast (p less than 0.0001); and in CA IDC compared to CA normal breast (0.0002), but not in AA tumors compared to CA tumors using Mann-Whitney analysis. Likewise, MAP kinase kinase kinase 1 was significantly overexpressed in AA IDC compared to AA normal breast (p less than 0.0001); and in CA IDC compared to CA normal breast (0.0061), but not in AA tumors compared to CA tumors using Mann-Whitney analysis. Overexpression of these genes is predicted to promote carcinogenesis. The long-term goal of our study is to identify genes that may contribute to the higher breast cancer mortality rates in AA women and to identify possible clinical biomarkers for prognosis, treatment, and targets for therapy.

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P60-10: DECIPHERING GENE REGULATORY NETWORKS USING GENEACT AND PRI

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Background: Deciphering gene regulatory networks requires the systematic identification of functional cis-acting regulatory elements. However, identification and functional characterization of those regulatory elements governing differential gene expression has been hampered by the limited understanding of their organization and locations in genomes. Disruption of transforming growth factor β (TGF- β) signal transduction pathway is one of the key cellular events involved in pathogenesis of breast cancer. TGF- β is a key negative growth regulator in mammalian epithelial cells and acts to prevent tumor formation in vivo. Our goal is to elucidate TGF- β transcriptional networks operative in normal and breast cancer cells and identify TGF- β or Activin genes whose regulation is altered in tumor cells.

Brief Description of Methodologies: We use the DNA microarray technology to profile transcriptional responses to TGF- β and Activin A. To gain unique insights into the complexed biological data, we developed two computational tools to analyze the DNA microarray data.

Results to Date: We developed two web-based bioinformatics tools called GeneACT (<http://promoter.colorado.edu>) and PRI (<http://barcode.colorado.edu/pri/>) to aid visualization and extraction of common regulatory sequence elements in the promoters and 3'-UTRs that are conserved across multiple mammalian species. Using these tools we discovered that clusters of transcription factor binding sites that are absolutely conserved in order and in spacing across human, rat, and mouse genomes are frequently active sites of transcriptional regulation. We term these regions Pattern-defined Regulatory Islands (PRIs). We demonstrated that PRIs may represent a fundamental property of the architecture of cis-regulatory elements in mammalian genomes and this feature can be exploited to pinpoint critical transcriptional regulatory elements governing cell type specific gene expression. GeneACT and PRI were applied to analyze transcription profiles of normal human mammary epithelial cells in response to Activin A and TGF- β .

Conclusion: Our results show that TGF- β and Activin A elicit distinct transcriptional profiles in normal human mammary epithelial cells despite sharing of identical downstream signaling transducers. Our analysis revealed a distinct set of cis-regulatory elements may be involved in mediating TGF- β transcriptional responses. Development of bioinformatics tools is expected to facilitate researchers to identify transcriptional networks that may be altered in breast cancer cells.

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P60-11: GLOBAL SURVEY OF ALTERNATIVE SPLICING IN HUMAN CANCERS USING QUANTITATIVE MICROARRAY PROFILING

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We have recently described the development and application of a microarray platform for the global-quantitative analysis of alternative splicing (AS) in mammalian cells and tissues. Data from this platform has resulted in the identification of thousands of new tissue-specific AS events, AS events that display species-specific differences and sets of AS events that display functionally coordinated regulation in tissues and during cellular differentiation.^{1,2,3,4} More recently, we have used the platform to identify AS events that are differentially regulated in tumor tissues compared to the corresponding normal tissues. In an initial study focusing on comparisons of tumor and matched normal tissue from lung cancer patients, we have identified several AS events that display pronounced and consistent differences between these two sources. Interestingly, these AS events are found in genes that play critical roles in signaling pathways known to be deregulated in certain cancers.

To expand our AS profiling system and data, we have employed a new human-specific array design that affords the monitoring of a comprehensive set of cassette type exons that are conserved in mouse. Using this new design, we have generated data by hybridizing cDNA from over 50 normal human tissues, including breast tissue, and from a range of cell lines including lung, colon, and breast cancers. This dataset will be complemented by profiling cDNA from additional primary tumor samples.

Using data generated from the new profiling work, we aim to discover additional splice variants that display significant differences between cancer-derived and normal tissues and between specific cancer types. By analyzing sets of AS events that display similar patterns of deregulation, we also aim to identify sequence elements and corresponding transacting factors that are associated with altered splicing patterns in cancers. This work should significantly advance our understanding of the role of splicing in human cancers.

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P60-12: INDUCTION MECHANISMS OF SECRETORY CLUSTERIN IN BREAST CANCER: A PRO-SURVIVAL PROTEIN REGULATED BY IGF-1 AND p53

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Breast cancer is the second leading cause of cancer death among women. There are several strategies to treat breast cancer, and one of them is ionizing radiation (IR) therapy. Treatment of this disease remains a very challenging goal. Due to the nature of the disease, many human breast cancers remain resistant to IR, presumably due to their abilities to recover and repair IR-induced damage. It is, therefore, important to understand the molecular events that occur in both the tumor and surrounding normal tissues to enhance the efficacy of therapy, which will presumably involve the inhibition of these repair and recovery processes.

Prosurvival proteins that are induced by IR can promote resistance of tumor cells. We theorize that induction of one such protein, secretory clusterin (sCLU), is involved in cancer progression, being elevated in metastatic breast cancers, and known to prevent tumor cell death. We determined that both basal and IR-induced expression of sCLU is

dependent on insulin like growth factor 1 (IGF-1) signaling in MCF-7 cells. IGF-1 is upregulated in many neoplasms including cancers of the breast, and during breast cancer progression, similar to sCLU. The overall goal of our project is to understand the regulatory mechanisms controlling expression of IGF-1 and therefore downstream sCLU expression, which we believe will be key to developing new treatments to enhance the radiosensitivities of tumor cells.

Our recent data suggest that sCLU expression is regulated by the stress-induced synthesis of the IGF-1 ligand. We hypothesize that activation of sCLU after IR and repression by p53 is, in fact, directly due to the regulation of IGF-1 in breast cancer cells in the following manner: (a) IGF-1 is upregulated after IR and (b) p53 suppresses IGF-1 transcription causing a downstream change in sCLU levels. We have been able to show IR-induced expression of IGF-1 ligand and IGF-1 promoter activity. However, the extent of the IR induction is dependent on wild-type p53 expression. Cells that are positive for wild-type p53 have minimal induction of the IGF-1 promoter compared to cells that are knocked down for p53. To further explore our hypothesis, we are examining the regulation of IGF-1 by analyzing the IGF-1 promoter, as well as IGF-1 mRNA and protein expression, after altering p53 function. In addition, we are functionally analyzing the promoter sequences of the IGF-1 promoter and have identified NF-Y transcription factor binding sites, which may play a role in the p53 regulation of IGF-1. We are also in the process of investigating the transcription factors that may be involved in the IR induction of IGF-1. We will then identify and confirm the transcription factors that are involved in this regulation through promoter DNA pulldown and ChIP assays. These studies will lead to determination of other cellular targets that may modulate expression of IGF-1-sCLU signaling in cancer cells, enhancing the efficacy of IR in breast cancer.

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P60-13: CELL ADHESION REGULATES KAIISO EXPRESSION AND FUNCTION IN HUMAN MAMMARY EPITHELIAL CELLS

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Despite increased awareness and improved methods of detection, approximately 40,000 women in the United States die from metastatic disease every year. Altered epigenetic control of specific gene targets plays a role in the initiation and progression of many types of cancer. One mechanism influencing such changes involves aberrant cytosine methylation in promoter-associated CpG islands. DNA methylation has a role in mediating expression of a variety of genes during normal mammalian development and tissue differentiation. In cancer, altered DNA methylation patterns are associated with abnormal gene expression, resulting in loss of expression of tumor-suppressor genes as well as inappropriate expression of genes with roles in specialized cellular transitions. Methyl-CpG-Binding Proteins (MBP) comprise a family of proteins that associate with regions of methylation and function to control transcription in a promoter specific manner. It is unclear what factors control promoter selectivity; however, certain MBP have promoter specific association patterns and their altered expression has been observed in breast cancer. We surveyed expression of MBP proteins (MBD2, MBD3, MeCP2, and Kaiso) across 9 human breast cell lines and identified Kaiso as the most differentially expressed MBP between normal and tumorigenic cells. Kaiso, unlike other MBP family members, is known to be inhibited by p120-catenin binding, an interaction that suggests cross-talk between Kaiso and E-cadherin (CDH1). CDH1 is an important tumor suppressor protein essential for proper epithelial cell maintenance, and loss of functional CDH1 adhesion results in increased invasion and metastasis both in vivo and in vitro. While the normal mammary epithelial cell lines (MCF10A and MCF12A) retained strong Kaiso expression, a loss of protein expression was observed in all breast cancer lines examined. Kaiso expression in the normal cell lines was significantly down-regulated at confluency, and examination of cultures at varying cell densities suggests a mechanism of cell adhesion dependent regulation. Disruption of cell adhesion by trypsinization resulted in an immediate down-regulation of p120-catenin and an up-regulation of Kaiso levels within 1 hour. Significantly increased Kaiso expression was observed for 24 hours and then gradually decreased until confluency (96 hours). p120-catenin expression remained low for 4 hours and then increased to pre-trypsinization levels. These changes were associated with a transient down-regulation of Kaiso targets, including CCND1 and MTA2. Preliminary studies suggest that CDH1 adhesion may play a dual role in regulation of Kaiso and Kaiso-mediated targets, influencing Kaiso expression levels as well as modulating p120-catenin. Altered Kaiso signaling, through either disrupted CDH1 adhesion or loss of Kaiso expression, may affect a variety of targets relevant to breast cancer initiation and progression. Ongoing experiments are exploring the role of CDH1 adhesion on Kaiso regulation and function in normal breast cell lines as well as the effects of Kaiso re-expression in breast cancer cell lines.

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P60-14: Kaiso-MEDIATED TRANSCRIPTIONAL REGULATION OF THE CELL CYCLE REGULATOR, CYCLIND1 AND ITS EFFECT ON TUMORIGENESIS

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The Armadillo catenins, β -, γ -, and p120^{cas}, are multifunctional proteins implicated in cadherin-mediated cell adhesion, signal transduction, and transcriptional regulation of tumor-promoting genes. p120^{cas} interacts with Kaiso, a member of the POZ-zinc finger family of transcription factors implicated in tumorigenesis¹. Kaiso itself has been shown to be mislocalized in the tumor microenvironment, suggesting possible changes in regulation of its target genes². Kaiso has bi-modal DNA-binding and transcriptional repression properties; it recognizes and binds a sequence-specific Kaiso binding site (KBS), TCCTGCNA, and methylated CpG-dinucleotides via its zinc finger domain³. One of Kaiso's putative target genes encodes the cell cycle regulator, CyclinD1. CyclinD1 is one of the regulatory subunits of the cyclin dependent kinases (CDKs) that play an important role in cell cycle progression and proliferation. Using *cyclinD1* luciferase-reporter assays, we found that Kaiso represses transcription of the *cyclinD1*-driven luciferase expression reporter in a sequence-specific and/or methylation-dependent manner. Electrophoretic mobility shift assays (EMSAs) showed that Kaiso binds to methylated as well as KBS containing portions of the *cyclinD1* promoter in vitro. In vivo binding of Kaiso to the *cyclinD1* promoter was also demonstrated using chromatin immunoprecipitation (ChIP) experiments. These data led to our hypothesis that Kaiso regulation of the *cyclinD1* promoter may be a key contributing factor of the perturbed cell cycle events that lead to tumorigenesis. To test this hypothesis, we are currently correlating *Kaiso* and *cyclinD1* expression in a tissue microarray of 1,000 clinically annotated human breast and ovarian tumor tissues. We are also performing qRT-PCR analyses of human breast tumor tissue. Finally, using stable Kaiso overexpression and depletion breast tumor cell lines, we will be assessing cell proliferation and CyclinD1 expression levels. Since *cyclinD1* overexpression is a common oncogenic event leading to tumorigenesis, the study of its regulation by Kaiso will lead to better understanding of CyclinD1's roles in tumorigenesis and contribute to the design of future therapeutics.

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P60-15: MODULATING SPLICING OF STEROID RECEPTOR RNA ACTIVATOR (SRA) ALTERS THE EXPRESSION OF GENES INVOLVED IN BREAST TUMORIGENESIS AND ESTROGEN RECEPTOR SIGNALING

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The steroid receptor RNA activator (SRA) has originally been identified as a noncoding RNA co-activating steroid receptors. Coding SRA transcripts have now however been characterized, encoding for a steroid receptor RNA activator protein (SRAP), which also modulates steroid receptor activity. *SRA1* gene products have therefore the unusual property to act as RNA as well as protein. The balance between these two genetically linked entities is controlled by alternative splicing of *SRA1* intron-1, whose retention alters SRAP reading frame. Herein we explore our ability to alter the balance between coding and noncoding transcripts by transfecting an antisense oligoribonucleotide to reprogram *SRA1* intron-1 splicing. We first establish the proof-of-principle of our approach using a minigene strategy and further demonstrate that oligoribonucleotide treatment of T5 breast cancer cells increased the endogenous levels of SRA transcripts retaining intron-1. Reprogramming SRA splicing modifies not only the expression of genes such as the urokinase plasminogen activator, known to participate in invasion processes, but also the response of cells to estrogen, a hormone intimately involved in breast tumorigenesis. This suggests that controlling the generation of non-coding and coding SRA RNAs might provide a new window of opportunity to fight hormone-dependent diseases such as breast cancer.

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P60-16: HIGH-THROUGHPUT BISULFITE SEQUENCING ANALYSIS OF THE CANCER EPIGENOME BY 454-SEQUENCING

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Although epigenetic components are being found to play a major role in driving tumor initiation and progression in human cancers, the methylation landscape in the cancer epigenome is still largely unexplored. Systematic sequence-based methylation analyses are notably absent and as a result, the potential diagnostic values of specific methylation differences in cancers remain largely untapped. By identifying the aberrant methylation "hot spots" in the cancer epigenome, we can target these genes for therapeutic intervention and develop them into DNA methylation biomarkers for early detection, diagnosis, prognosis, and monitoring the response to therapy. The integration of microarray technologies has significantly improved the scale and throughput of DNA methylation analyses. However, most of these existing methods only survey the presence or absence of methylated DNA but provide little detail about the extent and pattern of cytosine methylation. Therefore, despite the recent advances in epigenomic research, a high-throughput quantitative method capable of genome-wide, single methyl-cytosine resolution analyses is still unavailable. Toward this end, we generated a large-scale bisulfite sequencing approach for analyzing genomic methylation patterns by combining bisulfite treatment of genomic DNA with Roche-454 FLX ultra-high-throughput sequencing. Genomic DNA from cancer cell lines was digested with three restriction enzymes (*Csp* 6I, *Bfa* I, and *Mse* I) and ligated with adapters. The methylated DNA fragments was isolated and enriched from the adaptor-ligated genomic DNA using a protocol called Methylated-CpG island recovery assay (MIRA), which is based on the high affinity of a complex of the MBD2b and MBD3L1 proteins for methylated DNA. The methylation-enriched genomic library was then treated with bisulfite amplified by PCR with primers designed to amplify molecules carrying bisulfite-modified adapter sequences at both ends. The PCR products were sequenced using the GS FLX sequencer. We have so far collected 94,367 bisulfite sequences (approximate 16.3 Mb) with an average read length of 176 bp (range 45 bp to 469 bp) and more are on the way. This approach, unlike previous methods, allows us to conduct single-molecule bisulfite sequencing analyses on a genome-wide scale. This has led to the determination of methylation on previously undiscovered components of the genome along with a detailed analysis of the sequence composition and distribution of genome methylation. This new generation of methylome sequencing will provide digital profiles of aberrant DNA methylation for individual human cancers and offers a robust method for the epigenetic classification of tumor subtypes.

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P60-17: THE ROLE OF ALTERNATIVE SPLICING IN BREAST CANCER PROGRESSION

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Changes in alternative pre-mRNA splicing are associated with multiple types of cancer, including breast cancer. However, two important questions remain to be answered. First, do these changes in splicing actually contribute to cancer? Simplistically, are they "causes" or merely "symptoms" of tumorigenesis? At least some cancer-specific splicing variants appear to make important functional contributions to the transformed state, such as inhibiting apoptosis or blocking tumor suppressor activity. The second question concerns the character of the splicing change itself. Does the alternative splicing pattern derive from a regulated switch, like tissue-specific splicing, or does it result from a loss in splicing accuracy? For example, breast cancers show a large increase in alternatively splicing, whereas in normal cells such failures occur infrequently. We have addressed the second important question and tested the hypothesis that the accuracy of the spliceosome is compromised in breast tumor cells. First, we performed experiments to determine the accuracy of pre-mRNA splicing. Three important conclusions could be drawn from our observations. The spliceosome recognizes and removes introns with an astonishingly high degree of accuracy that is limited by the quality of pre-mRNAs generated by RNA pol II. Second, alternative splicing is a feature of all multi-intron pre-mRNAs resulting in mRNA isoforms that represent all possible exon ligation combinations. Third, the high levels of alternative splicing observed in the human genome are the consequence of sub-optimal splicing signals.

After establishing a reliable and reproducible protocol to assess the accuracy of pre-mRNA splicing, we proceeded to compare the splicing fidelity of cell lines derived from breast cancer and matched control cell lines. The results demonstrated that the accuracy of pre-mRNA splicing is not compromised in breast cancer cell lines. We conclude from these experiments that alternative splicing that accompanies cancer progression is not caused by global mis-regulation of the spliceosomes. Instead, alternative splicing in breast cancer derives from regulation, like tissue-specific splicing. Our current work investigates how breast cancer specific pre-mRNA splicing is mediated.

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P60-18: FORKHEAD BOX PROTEIN A1 ACTIVITY IN BREAST CANCER CELLS IS REGULATED BY SUMOYLATION

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Background: Estrogens and estrogen receptors (ER) regulate the growth potential of breast cancer cells (BCC). Recent genome-wide chromatin immunoprecipitation and bioinformatics studies have shown that the forkhead box transcription factor A1 (FoxA1) is required for binding of ER to the regulatory regions of a subset of ER target genes and expression of these genes. Additionally, estrogen-induced reentry of breast cancer cells into cell cycle requires FoxA1, suggesting an important role for FoxA1 in breast tumor growth. We have found that FoxA2, a neuronal and endocrine tissue-enriched FoxA family member highly related to FoxA1, is posttranslationally modified by covalent attachment of small ubiquitin-related modifier peptide (SUMO) in insulinoma cells. FoxA2 protein stability and activity is regulated by sumoylation.

Objectives: The objectives of this study were to: (1) determine whether FoxA1 is posttranslationally modified by sumoylation in BCC; (2) examine the effect of interfering with FoxA1 sumoylation on FoxA1 protein expression; (3) assess the effect of sumoylation on FoxA1-mediated gene expression.

Methods: FoxA1 was cloned into HA epitope-tagged CMV-based expression vector. PCR-based mutagenesis was used to mutate the SUMO acceptor sites in FoxA1 and to inactivate the SUMO ligase activity of protein inhibitor of activated STAT1 (PIAS1). Sumoylation of FoxA1 was demonstrated by transfecting MCF7 and MDA-MB-231 cells with FoxA1 expression vector along with SUMO-1, SUMO-2, SUMO-3, and/or wild-type or mutated PIAS1. FoxA1 was immunoprecipitated and analyzed by western blotting with SUMO or epitope tag-specific antibodies. Effect of sumoylation or lack of it on the transactivating activity of FoxA1 was analyzed by transfecting MCF7 cells with p27Kip1 promoter-luciferase reporter along with wild-type FoxA1 or SUMO acceptor site mutated FoxA1 or SUMO-1 fused FoxA1 with or without SUMO-1.

Results: Immunoprecipitation and western blotting analysis showed that FoxA1 is sumoylated in MCF-7 and MDA-MB-231 cells. FoxA1 sumoylation was promoted by the wild-type PIAS1 but not by the SUMO ligase-deficient C350S mutant, PIAS1. Analysis of the FoxA1 protein sequence identified two potential sumoylation sites. Lysine to arginine substitution of the conserved lysine (K6) abolished FoxA1-sumoylation, suggesting that the K6 is the primary sumoylation site. In transfection experiments, FoxA1 induced activation of the p27Kip1 promoter activity was downregulated by SUMO-1, demonstrating that SUMO-1 negatively regulates FoxA1 activity. Fusing SUMO-1 inframe with FoxA1 abrogated the FoxA1-mediated activation of p27Kip1 promoter confirming negative regulation of FoxA1 transcriptional activity by SUMO-1. The nonsumoylatable mutant of FoxA1 (FoxA1K6R) activated the p27Kip1 promoter to a lower extent compared with the wild-type FoxA1. Together, our results demonstrate that SUMO-modification regulates the transcriptional activity of FoxA1.

Conclusions: Results show that FoxA1 is modified by sumoylation on lysine, K6, and the SUMO modification of FoxA1 modulates the activity of FoxA1 on its target gene promoters such as p27Kip1. Our results suggest that the sumoylation pathway and its breast cancer associated target, FoxA1, may serve as targets for breast cancer therapy.

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P60-19: ROLE OF p68 & p72 RNA HELICASE IN BREAST CANCER

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Background: p68 and p72 are homologous RNA helicases that are involved in RNA splicing, interference, and transcription. Moreover, our data indicate that they are overexpressed in breast and colon cancers, suggesting that they are potential oncoproteins. One way how p68 and p72 might promote tumorigenesis is through the inhibition of p21 transcription, possibly by interacting and thereby repressing the transcription factor Miz-1. In addition, p68 and p72 are posttranslationally modified by acetylation, yet the functional significance of this posttranslational modification has remained unresolved.

Objectives:

1. To study how acetylation affects p68 and p72
2. To analyze how p68/p72 and Miz-1 regulate tumor suppressors p15, p21, and p57
3. To determine the role of p68/p72 overexpression on breast tumorigenesis in vivo

Methodologies: By site-directed mutagenesis and mass spectrometry, we have determined the sites of acetylation in p68 and p72. Pulse-chase experiments were used to investigate the impact of acetylation on the half-life of p68/p72. Further, foci formation and soft agar assays were utilized to study the impact of acetylation on the transforming properties of p68/p72. Reporter assays with an estrogen-responsive luciferase gene were employed to uncover the role of acetylation on p68/p72-dependent transactivation. MMTV (mouse mammary tumor virus)-p68 and MMTV-p72 constructs were injected into pronuclei of fertilized mouse eggs that were implanted into foster mothers. Resultant progeny was tested by PCR for transgene integration.

Results to Date: We have identified six and three acetylation sites in p68 and p72, respectively, and constructed point mutants that abolish acetylation. Our results suggest that acetylation may affect half-life and transactivation potential of p68 and p72. However, we could not reproduce published data that p68/p72 have transforming properties and thus could not assess if this is affected by acetylation. Furthermore, we have successfully established five (p68) and two (p72) germline-transmitting transgenic mouse lines. These mice are currently aged to observe whether breast tumorigenesis is induced by p68/p72 overexpression.

Conclusions: The establishment of p68/p72 transgenic mice will allow us to answer the questions if p68/p72 are oncoproteins in their own right or if they augment breast tumorigenesis induced by other oncoproteins, including HER2/Neu. If so, we would have established that p68/p72 are not only breast tumor markers but also valid pharmaceutical targets. In this case, our transgenic mouse models might be useful for testing novel drugs aimed at the eradication of breast cancer. Furthermore, our analysis of p68/p72 acetylation will provide valuable insight into the modulation of these RNA helicases. Moreover, affecting acetylation of p68/p72 by focusing on enzymes that execute this posttranslational modification may suggest another way of how to inhibit p68/p72 and thereby treat breast cancer.

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P60-20: ACTIVATION OF CELLULAR IRES-MEDIATED TRANSLATION BY eIF4G IN VIVO

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A variety of mRNAs encoding growth factors and oncogenic proteins feature 5' untranslated regions (UTRs) that enable cap-independent, internal ribosomal entry site (IRES)-mediated initiation of translation. This mode of initiation is facilitated by alternative means of ribosome recruitment to the mRNA. Here we provide evidence that eukaryotic initiation factor (eIF) 4G, plays a fundamental role in regulating c-myc and vascular endothelial growth factor (VEGF) IRES activity in vivo. IRES-dependent translation was investigated in cell lines that inducibly express either full-length eIF4G or its C-terminal fragment (Ct) lacking interaction with eIF4E and poly(A) binding protein (PABP). Expression of Ct led to specific activation of cellular but not viral IRES-mediated translation. The stimulatory effect of Ct was not due to loss of PABP or eIF4E interactions. Instead, our data suggest altered affinity of Ct for cellular IRES-containing mRNAs as the underlying mechanism for translation enhancement.

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P60-21: TRANSFER RNAS AS BIOMARKERS AND REGULATORS OF GENE EXPRESSION IN BREAST CANCER

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Gene expression is tightly controlled at many levels. Transfer RNAs (tRNAs) read mRNA codons during translation and its abundance and type can be important in translational regulation. The NIH reference human genome contains an unexpected large diversity of tRNA genes: over 270 unique tRNA sequences (isocodons) are present among ~450 tRNA genes. Human tRNA genes are further divided into 49 isoacceptor families that read the 61 codons in the genetic code. Varying the expression levels of tRNA isoacceptors could affect the translational efficiency of key genes required for the tumorigenic process based on their codon usage. From the biomarker perspective, each breast cancer cell type may exhibit a unique expression pattern of tRNA isocodons.

Due to the extensive secondary structure and the presence of a large number of post-transcriptional modifications, genome-wide analysis of tRNA expression is technically challenging. We previously developed a microarray method for the analysis of human tRNA identity and abundance. tRNA expression is significantly different in human tissues and this difference can be useful in the translational control of tissue-specific genes [1]. This "first-generation" array relies on hybridization alone to distinguish human tRNAs. Only 27 of the 49 isoacceptor families can be uniquely measured. We are working on a "second-generation" human tRNA array capable of comparative measurement of all tRNA isoacceptor families. We are using these microarrays to look for correlations between tRNA isoacceptors and mRNA expression levels in breast cancer cell lines to assess the role of tRNA in translational regulation. We are also working on a "third-generation" human tRNA array capable of comparative measurement of a majority of human tRNA isocodons. We will use these microarrays to profile tRNAs in breast cancer cells as potential biomarkers.

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GENOMICS AND PROTEOMICS II

Poster Session P61

P61-1: EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY ANALYSIS IN BREAST CANCER SUBTYPES

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The epidermal growth factor receptor (EGFR/HER1) pathway is a complex signaling cascade that regulates proliferation, apoptosis, differentiation, mobility, and adhesion. In breast cancer, the gene and protein expression of EGFR varies across the intrinsic gene expression subtypes; EGFR protein is high in 50% of basal-like tumors but is rarely observed in estrogen receptor (ER)-positive tumors. These observations suggest that there are differences among the breast tumor subtypes in regard to their sensitivity to EGFR inhibitors and EGFR-associated signaling patterns.

To investigate this hypothesis, we used a panel of basal-like and luminal cell lines to determine sensitivity to two EGFR inhibitors, gefitinib and cetuximab. The basal-like cell lines were 2- to 100-fold more sensitive to gefitinib. Only a single cell line was sensitive to cetuximab, which was the basal-like SUM102 line. The basal-like tumor-derived cell lines were also more sensitive to carboplatin compared to the luminal cell lines, and the combination of cetuximab and carboplatin was synergistic. A randomized Phase II clinical trial (NCT00232505) performed in part at the University of North Carolina examined the effect of cetuximab with and without carboplatin in patients with basal-like breast tumors. Benefit was not observed in the cetuximab-only arm, but the combination arm did have responders and stable disease. The final trial results are expected to be reported in 2008.

The disparity both in EGFR expression distribution and sensitivity to EGFR inhibitors in the basal-like and luminal cell lines led us to examine gene expression patterns associated with EGFR activation across the intrinsic subtypes. An EGFR-associated activation signature was developed in vitro and evaluated on 241 primary breast tumors and 7 normal breast samples. Three distinct clusters of genes were identified in vivo, of which two were predictive of poor patient outcomes in two independent data sets. While only 50% of basal-like tumors show high protein expression of EGFR, greater than 95% of basal-like tumors showed high expression of at least one of the EGFR-activation clusters. Most of the luminal B and HER2A tumors also had high expression of at least one of the EGFR-associated signatures even though they did not have high expression of EGFR itself. Pathway analysis of gene expression data from each subtype identified relationships with important mechanistic implications and highlighted the differences across the subtypes. Chi-square analysis found that high expression of many genes downstream of EGFR particularly in the MEK-ERK pathway (like KRAS and CRYAB) were correlated with the basal-like subtype. These data suggest that there may be multiple mechanisms downstream of EGFR that can activate EGFR-RAS-MEK pathway signaling. Inhibition further down in the pathway such as by MEK inhibitors may be an effective targeted therapy for the basal-like subtype.

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P61-2: IDENTIFICATION OF NOVEL TARGETS FOR THE TREATMENT OF ESTROGEN RECEPTOR-NEGATIVE BREAST CANCER

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Breast cancer is the number one cause of death in women between the ages 40 and 55. About 60% of breast cancer is estrogen receptor (ER)-positive and can be effectively treated with selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs). ER-negative cancers, however, have no specific, effective treatments. The signaling pathways that govern ER-negative cancer growth are poorly described. It is our hypothesis that genomic technologies can identify those genes that are responsible for ER-negative tumor growth. Using Affymetrix gene expression profiling on 2 independent sets of human breast tumor samples with known ER, PR, and HER2 status, we molecularly profiled breast tumors and identified a list of kinases that were differentially expressed in ER-negative tumors. Using the known and putative human "kinome," we identified 70 kinases in the first set of 43 tumors that were more highly expressed in ER-negative breast tumors than in ER-positive tumors (>2.3 fold, p-value <.05). The second set of 59 independent tumors identified another 84 kinases meeting these criteria. The intersection of the kinase lists contained 37 genes in common. Unsupervised clustering analysis in both sets identified kinases that defined ER-negative, HER2 positive tumors as well as genes that identified ER-negative, HER2 negative tumors. Gene ontology enrichment analysis reveals a significant enrichment for genes involved in mitogenesis, cell cycle progression, cell differentiation, and cell migration. Validation of these kinases was confirmed using quantitative RT-PCR (Q-RT-PCR) in a group of breast cancer cell lines and an independent set of 60 human tumor samples. Additionally, publicly available data sets confirmed kinase overexpression in ER-negative tumors. Promoter analysis of kinases that have similar expression patterns identifies a common motif known to bind the E2F4 transcription factor. This suggests that E2F4 may be important in regulating expression of this cluster of kinases. Additionally, analysis of tumors with clinical follow-up show that high expression of kinases in the proliferation cluster confers a poor prognosis. To better understand the biological

relevance of kinase overexpression, we used siRNA knockdown of individual kinases and found that several of the kinases were critical for ER-negative breast cancer cell growth. Based on these preliminary results, a Phase II clinical trial on women with ER, PR, HER2 negative ("triple-negative") breast cancer is currently being conducted using a multi-kinase inhibitor to the kinases identified in this study. We conclude that gene expression microarray analysis is a robust means of identifying kinases upregulated in ER-negative breast tumors and have identified specific kinases that are highly expressed in ER-negative breast cancers as compared to ER-positive breast cancers. Furthermore, Q-RT-PCR analysis confirms that these kinases are also elevated in ER-negative breast cancer cell lines and independent sets of human breast tumors. Finally, knockdown of expression of certain kinases using siRNA technology inhibits proliferation of ER-negative cell lines with no effect on ER-positive breast cancer cell growth, suggesting that signaling through pathways involving these kinases may be necessary for ER-negative breast cancer growth. These kinases may serve as druggable targets for the treatment of ER-negative breast cancer.

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P61-3: IDENTIFYING DISTINCT MULTI-ETHNIC GENOME-WIDE ALTERATIONS IN BREAST CANCER USING PARAFFIN EMBEDDED SAMPLES

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Background and Objectives: It is recognized that ethnic-specific disparities in stage of presentation/survival rates exist in breast cancer (BC) patients. These disparities remain an enigma. To investigate possible genetic contributions to these disparities, we are extending our previous study of genomic changes in BC samples from African American (AA) women to a multi-ethnic cohort consisting of 20 each AA, His (His), and non-Hispanic white (Cauc) women matched for age of diagnosis, cancer stage, and hormone receptor status. A main study goal is to identify differentially expressed genes between tumor and normal tissue that are common or unique among the three ethnic groups.

Methods: Tissue samples are evaluated for gene expression differences as well as DNA copy number (CNV)/chromosome alterations by high-density SNP arrays. We present here our most recent results of investigations focusing on triple-negative (ER-/PR-/Her2-) patients. Pathology specimens were freshly cut from FFPE blocks and marked by a pathologist as to normal versus tumor tissue. Almac Diagnostics performed RNA isolation, labeled cDNA preparation, and hybridization of tumor and normal cDNAs to a breast cancer-focused gene expression microarray (*Breast Cancer DSA Research Tool*). Each patient had self-matched gene expression studies (tumor versus normal). Using the FFPE samples, to date, approximately 17,516 transcripts were expressed on the *Breast Cancer DSA* with intensity significantly higher than background. Approximately 18,000 transcripts were evaluated and analyzed for fold changes in expression differences in normal versus tumor tissue. Distribution analysis, hierarchical clustering, and principal component analysis were used to analyze the data sets among patients within and across ethnic groups.

Results: For the normal tissue samples, 9,399 transcripts were detected in all three ethnic groups, while in tumor tissue samples, 10,296 transcripts were detected. There were also selected transcripts (hundreds to a thousand) that were detected in one or two ethnic groups only. Using two-way ANOVA (disease state and ethnicity) and a p-value cutoff of 0.01, a subset of 6,479 highly consistent/significant genes was selected and further used in data quality control. Data QC indicated patient samples clustered well with respect to both ethnicity and normal versus tumor tissue. From these analyses of this limited sample set, we have already identified ethnic-specific expression patterns in tumor specimens as well as in matched normal tissue samples. We are completing these studies mapping clusters of differentially expressed genes into pathway analysis, validation by real-time PCR, and genome-wide CNV studies. We will present our latest findings.

Conclusions: The ultimate aim of the completed study is an increased understanding of the biological basis of ethnic-specific BC disparities, leading ultimately to individualized, ethnic-specific diagnostic and therapeutic approaches.

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P61-4: IDENTIFICATION OF 18Q TRANSCRIPTS DELETED IN BREAST CANCER

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Array comparative genomic hybridization experiments were performed on breast cancer specimens using genomic arrays covering 18q21-q23. A novel homozygous region of loss at 18q22.3 was detected in 50% of breast tumors. This region of loss was also

detected in prostate tumors, but not in tumors from 10 other organ sites. There are no known genes located within this region.

Since chromosomal regions exhibiting homozygous deletion are not commonly found and are usually the site of tumor suppressor genes, we proposed the following hypothesis: Encoded within the region of homozygous deletion at 18q22.3 is a transcript(s) that plays a role in the development or progression of breast cancer. To test our hypothesis, we proposed to: (1) develop a custom oligonucleotide microarray covering this region and hybridize the array with fluorescently labeled cDNA that will be obtained by reverse transcribing RNA isolated from primary mammary epithelial cells and (2) confirm and analyze the identified transcript(s).

For aim 1, we have developed an oligonucleotide microarray tiled every 10 basepairs covering the 18q22.3 deleted region that is being synthesized by Agilent Technologies. RNA has been isolated from the commercially available human primary mammary epithelial cell line HMEC (Lonza) and converted to cDNA. We are in the process of fluorescently labeling the cDNA and hybridizing it to the microarray. For aim 2, we will confirm and characterize full-length transcribed sequences that are identified from the oligonucleotide array experiment. Following the confirmation and characterization of the transcribed sequences, commercially available breast cancer cell lines will be analyzed to determine if the region is deleted and to determine the expression level of the transcript(s) in the cancer cells. Through these experiments, we hope to identify a novel molecular pathway that is altered in breast cancer cells that could provide new targets for developing therapeutics.

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P61-5: LONG, ABUNDANTLY EXPRESSED, NON-CODING TRANSCRIPTS ARE TARGETS OF ALTERATION DURING THE DEVELOPMENT OF BREAST CANCER

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The proportion of the human genome that is responsible for encoding protein sequences is less than 2%. In spite of this, the majority of the focus in cancer research has been on this small proportion of the human genome and how alterations to it participate in cancer development. Recent research using whole-genome tiling arrays have demonstrated that the transcriptional output of the human genome vastly exceeds the much smaller proportion of protein-coding transcripts. Short regulatory non-coding RNAs (ncRNA) are but one small group of ncRNA species that are also comprised of longer ncRNA species with potentially their own unique properties. We used tiling arrays to catalog the full transcriptional output of a human bronchial epithelial cell line. Our analysis from this study focused on a specific subset of non-coding transcripts (NCTs) displaying the following criteria: (1) between 400–3,000 nt in length, (2) abundantly expressed (at or near the level of beta-actin), and (3) highly conserved (greater than 95% sequence conservation between all species between humans and zebrafish). Such long (>400 nt) transcripts are all capable of profound secondary structures of unknown significance. Additionally, these long NCTs are not precursors of any known miRNAs, and we can detect abundantly expressed long transcripts corresponding to the putative size of these NCTs. There are alterations in the expression of these transcripts in both primary breast and ovarian cancers. Several of the breast cancers had mutations in these NCTs, and specific sequence alterations in the NCTs were repeatedly observed. To better characterize these NCTs we have initiated a detailed study on five of the most conserved long transcripts. Both RACE (5' and 3') and northern blots were used to determine the full length of each NCT. The results show that their lengths range from 450 nt to as long as 3,000 nt. We have utilized siRNA to decrease expression of two of these NCTs in both a normal breast epithelial cell line and a breast cancer-derived cell line. Phenotypic alterations (growth, apoptosis, and stress response) in siRNA-exposed cells were examined. Gene expression profiles (using Affymetrix U133 Plus 2 chips) of cell lines both before and after siRNA was used to decrease expression of the NCTs were compared. From these data we specifically investigated NCT-linked genes involved in specific pathways associated to the development of breast cancer. Our hypothesis is that these long, highly conserved, abundantly expressed transcripts play an important regulatory role within cells and are a potentially important target for alterations in expression or sequence during breast cancer development.

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P61-6: HIGH-THROUGHPUT RNAi PHENOTYPIC SCREENING FOR THE IDENTIFICATION OF GENES THAT MODULATE ESTROGEN ACTIVITY IN HUMAN BREAST CANCER CELLS

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Breast cancer is the second leading cause of cancer-related death among women in the United States. Of the diagnosed cases of breast cancer, the majority are estrogen

receptor (ER) positive making the estrogen receptor a major target in breast cancer therapy. Although anti-estrogen therapy has proven successful in treating ER-positive breast cancer patients, acquired resistance of the tumor is known to occur. To develop more effective anti-estrogen therapies, our goal is to identify genes involved in modulation of the response to estrogen signaling in breast cancer cells using an innovative functional genomics approach based on high-throughput RNA interference (HT-RNAi) analysis. Assays for HT-RNAi were developed to monitor estrogen dependent signaling utilizing the T47D-KBluc breast cancer cell line, which expresses luciferase under the control of the estrogen response element. This cell line was adapted for HT-RNAi by optimizing siRNA transfection conditions in a 384-well format. Dose response curves were generated in assay format to determine the EC₅₀ for estrogen activity. The HT-RNAi assay involved the transfection of T47-KBluc cells with siRNA targeting 588 kinases and 24 hours later, treating the cells with an EC₅₀ dose of estrogen. The assay readouts consisted of parallel measurements of both ERE reporter activity and proliferation. Genes that modulate the response to estrogen are further validated using functional assays examining the gene's role in estrogen response. This approach will rapidly and efficiently identify important genes involved in estrogen-dependent growth of breast cancer cells including the identification of potentially novel drug targets.

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P61-7: PREDICTING MICRORNA-21 TARGET GENES IN MCF7 CELLS

Aaron Schetter

National Cancer Institute

MicroRNAs are potential diagnostic biomarkers and therapeutic targets for breast cancer. MicroRNAs are small noncoding RNA molecules that regulate the translation of many genes. MicroRNAs regulate a variety of biological processes including apoptosis, cell proliferation, differentiation, and carcinogenesis. Expression patterns of microRNAs are associated with clinical outcomes for lung, pancreatic, and colorectal cancers, indicating that altered microRNA levels may be involved in the progression of these malignancies. Previous work found microRNA-21 (miR-21) to be altered in all solid tumor types. Increased levels of miR-21 are found in breast tumor tissue, and it may play a role in breast carcinogenesis. Altering miR-21 has already been shown to affect cell proliferation and apoptosis in breast cancer cell lines, consistent for a role in the development of breast cancer.

To gain further insight into a role for miR-21 in breast carcinogenesis, we set out to experimentally identify targets for miR-21 in human breast cancer cell lines. Our strategy to identify targets was to alter miR-21 expression in MCF-7 cells and examine the gene expression pattern using microarrays. MicroRNAs inhibit expression of particular genes by guiding the RNA-induced silencing complex (RISC) to specific mRNAs based on sequence specificity. The RISC complex can then destabilize those mRNAs and prevent them from being translated. Overexpressing miR-21 should cause the RISC complex to bind and destabilize miR-21 target genes resulting in reduced gene expression as detected by microarrays. Conversely, inhibiting miR-21 may result in the increased expression of target genes.

Inhibition of miR-21 increased expression of 125 genes while overexpression of miR-21 reduced expression of 76 genes ($p < 0.005$). These are candidate targets of miR-21. Eleven of these genes contain predicted miR-21 binding sites, indicating that these 11 genes may be targets for miR-21. Altered expression of any of these genes may be a mechanism that miR-21 contributes to carcinogenesis. Among these 11 genes is HRAS, a gene with a known role in breast cancer. Six genes showed decreased expression upon overexpressing miR-21 and were expressed at higher levels when miR-21 was inhibited. This pattern is consistent with that of miR-21 targets, although none of these 6 genes are predicted to contain miR-21 binding sites based on current computer predictions. While it is possible that these 6 genes are targeted by miR-21, they may also represent consistent, secondary effects caused by the altered expression of miR-21. Among these 6 genes is transforming growth factor β 1 (TGF β 1). High miR-21 levels result in a reduction of TGF β 1 while reduction of miR-21 leads to higher levels of TGF β 1. TGF β 1 has been shown to act as a tumor suppressor gene in breast cancer models, and deregulation of TGF β 1 can enhance tumorigenesis. While TGF β 1 does not contain putative miR-21 binding sites, the downstream factor TGF β 1 (transforming growth factor B-induced) does contain miR-21 binding sites and is potentially a direct target for miR-21. Therefore, the increased expression of miR-21 in human breast tumors has the potential for inhibiting multiple members of the TGF β pathway, either directly or indirectly, providing a mechanism for the contribution of miR-21 in breast carcinogenesis.

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P61-8: miRNAS IN BREAST CANCER PROGRESSION**Richard W. Padgett,¹ Yi Sun,¹ Maocheng Yang,¹ and Michael Reiss²**¹State University of New Jersey, Rutgers and ²University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School

Background: Small, noncoding RNAs (microRNAs, miRNAs) are single-stranded RNA species of 19-23 nucleotides that are found in all animals. They function as regulatory molecules by binding to the 3'UTR sequences of target genes and inhibiting protein translation. Over 400 miRNA genes are predicted in humans and mice. From the limited number of studies, these miRNAs have been shown to be involved in a variety of important cellular processes, such as apoptosis, viral regulation, and developmental processes, and cancer growth. Because of their mode of action, they represent a new type of gene regulation and offer a unique opportunity to evaluate their involvement with breast cancers.

Objectives: Using a series of cell lines that define the progression of breast cancer from premalignant stages to metastatic stages, we profiled the expression of known miRNA genes. Our hypothesis is that the expression profile of a limited set of miRNAs increases or decreases as cells progress through the defined stages of breast cancer, and thus mutations in these genes will correlate and contribute to neoplasia. Further, the target genes that are regulated by these miRNA genes may constitute a novel set of genes, thus providing new potential targets for drug therapies. Our aims were to catalog miRNA expression profiles in the different stages of breast cancer, using a series of murine mammary cancer cell lines that represent the different stages of breast cancer. We expect that some miRNA genes may be associated with several independent breast cancer cell lines while others represent unique events.

Methods: In brief, RNA samples were collected from tissue culture cells using the Ambion mirVana™ kits that isolate small RNAs. Glass slides containing complementary oligos to all known mouse miRNA genes were hybridized and analyzed for expression patterns. Algorithms to predict target genes regulated by miRNAs has been developed and validated experimentally. Transfection protocols were used to introduce miRNA genes or target genes into mammalian cells for further evaluation of oncogenic or tumorigenic properties.

Conclusions and Results: Through our microarray experiments, we have shown that many miRNAs are differentially regulated as cells progress through cancer stages. These regulated miRNAs show at least six patterns of expression changes and may represent a regulatory network. A general trend in miRNA expression emerges from this work. As cells progress toward a metastatic state, more miRNAs are down regulated, rather than up regulated. This suggests that many cellular proteins are up regulated in these cells, and this could be involved in promoting tumor growth. We propose that the mouse is a good model system for the study of breast cancer since several miRNAs are similarly regulated in both mouse and human. Finally, we are beginning to gain insights into the mechanism of how miRNAs are involved in cell growth. We find that some of the misregulated miRNAs regulate cell death. The misregulation of cell death could allow cells to escape normal regulatory mechanisms for removing tumorous cells.

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P61-9: MICRORNA TARGET DETECTION AND ANALYSIS FOR GENES RELATED TO BREAST CANCER USING MDLCOMPRESS**Andrew Soliz Torres,¹ Scott Evans,² and Douglas Conklin³**¹GE Research and Development Center, ²GE Global Research, and ³State University of New York, Albany

We describe initial results of miRNA sequence analysis with the optimal symbol compression ratio (OSCR) algorithm and recast this grammar inference algorithm as an improved minimum description length (MDL) learning tool: MDLcompress. We apply this tool to explore the relationship between miRNAs, single nucleotide polymorphisms (SNPs), and breast cancer. Our new algorithm outperforms other grammar-based coding methods, such as DNA Sequitur, while retaining a two-part code that highlights biologically significant phrases. The deep recursion of MDLcompress together with its explicit two-part coding enables it to identify biologically meaningful sequence without needlessly restrictive priors. The ability to quantify cost in bits for phrases in the MDL model allows prediction of regions where SNPs may have the most impact on biological activity. MDLcompress improves on our previous algorithm in execution time through an innovative data structure and in specificity of motif detection (compression) through improved heuristics. An MDLcompress analysis of 144 overexpressed genes from the breast cancer cell line BT474 has identified novel motifs, including potential microRNA (miRNA) binding sites that are candidates for experimental validation in 3 genes (ESR1, PTGS2, and EGFR). These identified sequences, if validated, are potential predictive markers of breast cancer and therapeutic targets.

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P61-10: COORDINATE POSTTRANSCRIPTIONAL REGULATION OF CANCER GENES BY THE RNA BINDING PROTEIN HUR**Ulus Atasoy, Joseph Magee, and Matt Gubin**

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Due to the poor correlation between steady-state mRNA levels and protein products, traditional microarray analysis may miss many genes that are regulated primarily at the level of mRNA stability and translation. Posttranscriptional gene regulation is becoming increasingly recognized as an important form of cellular control. Whereas our understanding of transcriptional regulation is advanced, in contrast, this area remains largely unexplored. Yet, increasingly, the importance of microRNAs and RNA binding proteins (RBPs) is now beginning to be better appreciated. We study the *elav* (embryonic lethal abnormal vision) family of RBPs, which are paraneoplastic antigens, overexpressed in a variety of malignancies, including breast cancer. Antibodies against *elav* family members are believed to be cancer protective. The *elav* family of RBPs binds to the AU-rich elements (AREs) found in the 3' untranslated regions (UTRs) of many early-response genes, including proto-oncogenes and cell cycle regulators. HuR, the ubiquitously expressed family member, has recently been described to play a role in cancer progression. HuR stabilizes and translationally upregulates the expression of its target mRNAs. Elevated levels of cytoplasmic HuR directly correlate with increased invasiveness of malignancy and poor prognosis for many cancers, including those of the breast. HuR has been described to control the expression of genes in all six different areas of transformation originally described by Hanahan and Weinberg. Hence, it has been recently suggested that HuR may serve as a tumor maintenance gene that allows cancers, once they are established, to proliferate. Therefore, it is of interest to discover in vivo HuR targets, as these genes may play vital roles in transformed cells. We have developed methods that enable us to identify, en masse, in vivo targets of RBPs such as HuR from cell lines and now for the first time, solid tissues as well. We call this ribonomic analysis, in which we immunoprecipitate subsets of ribonuclear particles (RNPs), exact the mRNA, and then apply to microarrays. This technique, in which no cross-linking is used, allows one to en masse identify different in vivo mRNA targets that different RBPs interact with. Using a combination of HuR immunoprecipitation and RNA interference, we have been able to identify HuR in vivo targets that may play important roles in tumor maintenance. Furthermore, we present evidence of cross talk among stabilizer and destabilizer RBPs, which can potentially regulate the expression of multiple cancer targets. These techniques have the potential to better delineate genes whose steady-state mRNA levels may not significantly change but which are translationally active inside cancer cells. Potentially, the identification of such genes may offer novel therapeutic targets to inhibit cancer growth.

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P61-11: DETECTION OF GENES MODIFYING SENSITIVITY TO CHEMOTHERAPEUTICS USING AN shRNA LIBRARY IN BREAST CANCER**Gregory Hannon**

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Human tumors have great genetic diversity and in most types of cancer only a subset of patients will prove responsive to any given agent. Under selective pressure of a toxic therapy, the genetic diversity within most human tumors may lead to outgrowth of drug-resistant cells. Because of resistance to single agents, combination therapy is essential for tumor eradication. In the future, selection of individualized drug cocktails will be based on the molecular features of a patient's tumor resulting in more efficient treatment. By studying the molecular basis of chemotherapeutics, we will be able to identify subsets of patients that are more likely to respond to certain drugs thereby avoiding the needless cost and toxicity of ineffective treatment.

Simultaneously, recent advances in the field of RNA interference (RNAi) have enabled researchers to conduct in depth investigations of gene function. RNA interference is a biological phenomenon where many eukaryotic organisms respond to double-stranded RNA by activating a sequence-specific silencing pathway. Initiation of RNAi occurs upon processing of double-stranded RNAs into ~22 nucleotide fragments known as siRNAs by an RNaseIII nuclease called Dicer. These small RNAs are used as a guide for targeting and destroying complementary cellular mRNA through their incorporation into the RISC effector complex resulting in a specific loss of gene expression. Scientists can exogenously trigger RNAi in mammalian cells through the use of siRNA or short hairpin RNAs (shRNAs) allowing them to silence specific genes of interest. My research involves using this cutting-edge RNAi technology to study genes that modify sensitivity to chemotherapy.

High-throughput screens in cultured mammalian cells can now be performed due to the creation of extensive RNAi libraries such as our own Hannon-Elledge library. The application of RNAi has transformed the way we approach mammalian cell genetics. Over the past year we have made significant progress in several areas that have enhanced the use of shRNAs as a tool for genetic screens. Our second-generation shRNA retroviral library now covers 32,202 genes (86,128 clones) for human and

30,629 genes (76,896 clones) for mouse genomes. These shRNA can be delivered into cells, both in vitro and in vivo, using our optimized viral expression vectors. More importantly, with these tools we have successfully demonstrated the feasibility of performing genetic screens in mouse and human cells through our pilot efforts. Using the "synthetic-lethal" genetic approach, we can now perform drug-induced synthetic-lethal screens by using cancer drugs that are currently in clinical trials. This is important as cancer cells can often become resistant to the toxic effects of chemotherapeutics. Bortezomib (Velcade) is the first targeted therapeutic to the proteasome approved by the FDA for treatment against multiple myeloma and is currently in Phase II clinical trials for breast and lung cancers. Our goal is to identify genes that mediate resistance against Velcade that could serve as potential drug targets. RNAi technology is a powerful tool that could potentially be used to study and treat other human diseases through its application to mammalian cell genetics.

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P61-12: NEW GENOMIC STRATEGIES TO ACCELERATE THE DISCOVERY OF BREAST CANCER GENES

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Many genes across the cancer genome are altered during the cancer process. These changes provide selectable traits that drive the cellular evolution of neoplastic cells, and produce the malignant phenotypes that determine clinical outcome. Identification of new tumor suppressor genes in breast cancer genomes can therefore provide important insights into the pathobiology of the disease and enable the development of clinical interventions. Toward this end, we have developed and applied a variety of genome scanning technologies (such as array CGH) and integrated strategies (NMD/aCGH screening with HT re-sequencing) to identify tumor suppressor genes that are either structurally or functionally altered during the cancer process.

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P61-13: AN RNAi SCREEN IDENTIFIES ENHANCED TRIGLYCERIDE STORAGE AS A SURVIVAL FACTOR FOR BREAST CANCER CELLS WITH THE ERBB2 AMPLICON

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Overexpression of ERBB2 (Her2/neu) occurs in 20-30% of breast cancers and is a prognostic indicator for aggressive disease and reduced survival. Although well-known therapies have been developed targeting ERBB2, there is still a high recurrence rate of the disease. Recent molecular profiling studies have shown that there are approximately 150 genes that are co-overexpressed with ERBB2. A large-scale RNAi screen identified a number of genes that are necessary for the viability of the ERBB2-positive, BT474 cell line. Of the 19 best hits from this screen, five had known roles in lipid metabolism. Two of these genes, the PPAR γ binding protein (PBP) and the nuclear receptor subfamily 1, group D, member 1 (NR1D1 or RevErba), reside on the 17q12-21 amplicon, and encode transcriptional regulators of fat production and storage during adipocyte differentiation. NR1D1 has recently been identified as a major component of the circadian clock potentially linking the clock and cellular metabolism. RNAi against both genes resulted in apoptosis induction specifically in BT474 cells but not in MCF-7, MDA-MB-453, MDA-MB-468, normal breast cells (HMEC) or HEK 293 cells. NR1D1 knockdown also significantly decreased viability of another ERBB2-positive cell line MDA-MB-361. Although it is well established that ERBB2-positive breast cancer cells contain high levels of fatty acid synthase, we have found several lines of evidence that indicate that BT474 and MDA-MB-361 cells actively store synthesized fatty acids as triglycerides. Inhibition of the NR1D1 pathway interferes with this process and results in increased accumulation of the end product of fatty acid synthase, palmitate, and increased ROS formation consistent with palmitate lipotoxicity. Exogenous palmitate in combination with NR1D1 pathway inhibition had a synergistic effect on apoptosis induction and ROS formation in BT474 but not MCF-7 cells. Our results imply that amplification of genes from the 17q12-21 chromosomal region work in concert to supercharge fatty acid production. Increased activity of the NR1D1 pathway enables ERBB2 positive breast cancer cells to convert fatty acids to triglycerides allowing these cells to avert lipotoxicity.

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P61-14: HuR MEDIATED POST-TRANSLATION REGULATION OF PROLIFERATION RELATED GENES IN MCF-7 CELLS

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Recent advances in our understanding of gene expression have elevated the importance of post-transcriptional regulation in determining normal and disease cellular phenotypes. Central to these controls are RNA binding proteins (RBPs) that interact with regulatory elements present in specific groups or subsets of transcripts and choreograph their processing, splicing, transport, localization, stabilization, and translation. RIP-Chip is a powerful strategy that has emerged for studying these controls. This technology involves isolation of the subset of RNAs that interact with and therefore are controlled by specific RBPs. RBP-bound mRNAs frequently reveal functionally related genes that cannot be identified using standard gene expression approaches and provide novel insights into cellular biology.

We have applied RIP-Chip to investigate post-transcriptional regulation by HuR in a cellular model of breast cancer. HuR is a widely recognized regulator of cellular proliferation and has previously been implicated in a wide range of cancer phenotypes, including breast cancer. In the current studies, HuR was fused to a biotin acceptor domain and transiently expressed in MCF-7 cells that were treated with either vehicle or estradiol for 24 hours. HuR associated RNA was isolated using streptavidin affinity matrix and compared to polyA RNA obtained from the same samples using global microarray analysis. Using stringent criteria for differential expression, 105 genes were found to be differentially expressed upon estradiol treatment in the polyA samples. These genes mapped to a large (n=56) and diverse group of Gene Ontologies using GoMiner and making it difficult to identify critical components of the estradiol-mediated cellular proliferation without prior knowledge. Using similar criteria, 79 genes were found to be as differentially represented in the HuR subsets between vehicle and estradiol treatment. Of these, 62 were not found to be differentially expressed between the polyA samples and were unique to the HuR subset. Importantly, the majority of these genes (70%) contained AU-Rich Elements (AREs) to which HuR is known to bind. In contrast to the genes differentially expressed in the polyA samples on estradiol treatment, the genes unique to the HuR cluster mapped to a small (n=4) set of Gene Ontologies that were readily related to the known action of estradiol (e.g., negative regulation of cellular proliferation). These findings, their potential significance, and the potential application of these methods to the further study of breast cancer will be presented.

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P61-15: DIFFERENTIAL PATTERNS OF ALLELIC LOSS IN ESTROGEN RECEPTOR-POSITIVE INFILTRATING LOBULAR AND DUCTAL BREAST CANCER

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The two main histological types of infiltrating breast cancer, lobular carcinoma (ILC) and the more common ductal carcinoma (IDC), are morphologically and clinically distinct. Small studies using individual markers have demonstrated differences in patterns of allelic loss between the two subtypes. We conducted a whole-genome study using the Affymetrix GeneChip(R) Mapping 10K Array of 166 archival estrogen receptor (ER)-positive tumors (89 IDC and 77 ILC) to identify sites of loss of heterozygosity (LOH) that were either distinguished, or shared by, the two histologic subtypes. We found SNPs of high frequency LOH (>50%) common to both ILC and IDC tumors predominantly in 11q, 16q, and 17p. IDC tumors had a slightly higher frequency of LOH events across the genome than ILC tumors (Fractional Allelic Loss = 0.186 and 0.156, respectively). By comparing the average frequency of LOH by chromosomal arm, we found that IDC tumors had a significantly (p25%). Stratification by tumor size, mitotic rate, or DNA content, identified additional chromosomal arms differentiating ILC and IDC within groups of tumors with specific characteristics. Our results indicate a difference in the frequencies and locations of LOH events throughout the genome of ER-positive ILC and IDC breast tumors as well as fundamental difference in the dynamics of LOH events by histological subtype with regard to clinical characteristics.

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HORMONE RECEPTORS II

Poster Session P62

P62-1: DIFFERENT PROLACTIN RECEPTORS MEDIATE DIFFERENT FUNCTIONS IN BREAST CANCER CELLS SUGGESTING THE IMPORTANCE OF A SHORT AND A SOLUBLE FORM TO NORMAL BREAST HEALTH

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Prolactin (PRL) is a hormone that has been implicated as a contributing factor in the incidence and progression of breast cancer. Several different isoforms of the PRL receptor (PRLR) have been discovered, most of which are produced by alternative splicing of a single gene product. The major isoforms include a long form (LF), an intermediate form, and two short forms (SF1a and SF1b), each of which have the same extracellular sequence (comprised of S1 and S2 regions), but different intracellular signaling domains. We have discovered naturally occurring forms of each of these PRLRs, which maintain the same intracellular signaling regions, but lack about half of the extracellular domain. Importantly, these receptors proved to be constitutively active. The objective of our study was to use these constitutively active receptors (designated Δ S2) to determine the individual roles of the different PRLRs in mammary cells. In transiently transfected human breast cancer cells (T-47D), expression of Δ S2 LF increased cell number, whereas both Δ S2 LF and Δ S2 SF1a increased endogenous beta-casein gene expression. In addition, our data show the other short isoform, Δ S2 SF1b, inhibited cell proliferation and migration in a copy number-related fashion when stably overexpressed in several cell types. In an effort to further evaluate the effect of this apparently beneficial isoform, stable T-47D breast cancer cells expressing Δ S2 SF1b under the control of a tetracycline-responsive promoter were produced. Analysis showed that overexpression of Δ S2 SF1b induced prolonged ERK activation in the absence of ligand. Overexpression of Δ S2 SF1b also upregulated the cell cycle inhibitor, p21, and the milk protein, beta-casein, suggesting both an anti-proliferative and pro-differentiative role for this receptor, and by inference the regular SF1b receptor in the presence of ligand. In the course of our studies, we also identified another truncated PRLR isoform. Sequence analysis revealed an exon 6 deleted transcript. This deletion creates a frameshift in the open reading frame resulting in a foreshortened soluble receptor essentially composed of just half of the extracellular domain. This form, designated SS1 (for soluble S1), has been previously described at the mRNA level by Laud et al. (*Int J Cancer* 2000; 85:771-6). RT-PCR compared expression of transmembrane receptors to SS1 in tumor samples from patients with invasive ductal carcinoma versus histologically normal contiguous regions from the same patients. Preliminary results from four pairs showed a larger complement of SS1 in the normal regions, suggesting a beneficial role of this isoform. SS1 protein was detected in T-47D culture medium by immunoprecipitation. Co-immunoprecipitation demonstrated binding of PRL and SS1. Furthermore, SS1 conditioned medium, produced by the overexpression of SS1 in HEK293 cells, modulated PRL-induced signaling, prolonging ERK activation and reducing Stat5 activation. Thus far, the data suggest that SS1 is a soluble PRL binding protein that has the capacity to modulate PRL signaling. There is a correlation between loss of SS1 and the development of invasive ductal carcinoma, a result that suggests beneficial aspects to increased expression of SS1. Our data with Δ S2 SF1b supports the conclusion that increased expression of this, or the regular SF1b with ligand, would also be beneficial in breast cancer patients.

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P62-2: ELUCIDATING THE ROLE OF ESTROGEN-RELATED RECEPTOR α IN BREAST CANCER

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Background: Expression of estrogen-related receptor α (ERR α) correlates with poor prognosis in breast cancer. The three ERRs (α , β , and γ) are orphan members of the nuclear receptor superfamily. Despite significant homology to estrogen receptor, ERRs are unresponsive to natural estrogens, and no endogenous ligands have been identified. Of the three subtypes, ERR α is the best characterized and plays a role in physiologic processes as well as in prostate, ovarian, and breast cancers.

Several significant observations suggest that ERR α may be involved in breast cancer development and progression. First, ERR α is expressed more highly in breast cancer tissue than in surrounding normal tissue. Second, there is significant interplay between the ERR α and ER α signaling pathways; these receptors bind to similar DNA response elements in target genes and transcription of several endogenous genes can be activated by both receptors. Our primary objectives are (1) to determine the biological relevance of ERR α in breast cancer and (2) to identify the molecular mechanisms that are responsible for ERR α function in this setting.

Methods and Results: Without an available ligand to enhance the transcriptional activity of ERR α , it has been difficult to characterize specific ERR α target genes. Our lab has developed a customized coactivator to enhance ERR α activity without activating other nuclear receptor-mediated processes. In specific, we exchanged the nuclear receptor interacting motifs within the coactivator PGC1 α (peroxisome proliferator-activated receptor γ coactivator 1 α) with ERR α -selective interacting peptides. To identify novel ERR α target genes and to determine the extent to which the ER α and ERR α sig-

naling pathways impact each other, we expressed the ERR α specific PGC1 α in MCF7 cells in the presence or absence of estradiol. The resultant RNA was subjected to microarray analysis. From this analysis, we identified novel ERR α target genes that may contribute to the role of ERR α in breast cancer, including VEGF (vascular endothelial growth factor), CXCL12, and several GST (glutathione-S-transferase) family members. Additionally, we found that many ERR α target genes previously identified in muscle and liver cells can be induced by PGC1 α -coactivated ERR α in MCF7 cells as well. Many of these, which we have validated in a panel of breast cancer cell lines, are involved in the TCA cycle, oxidative phosphorylation and combating oxidative stress. Using RNAi to ablate ERR α expression, we confirmed that ERR α contributes to the basal expression of many of these factors.

Finally, to begin to define the biological role of ERR α , we tested the effects of altering its activity on breast cancer cell growth in vitro and in vivo. Our preliminary data suggest that depletion of ERR α by RNAi leads to a decrease in breast cancer cell migratory capacity in vitro. We are currently investigating the mechanism behind this phenotype and examining whether metastatic potential, as the in vivo correlate of migration, is similarly affected by ERR α signaling.

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P62-3: BREAST CANCER LYMPHATIC DISSEMINATION: INFLUENCE OF ESTROGEN AND PROGESTERONE

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Breast cancers commonly spread to lymph nodes (LNs). If the primary tumors are estrogen receptor (ER) and/or progesterone receptor (PR) positive, then the likelihood that LN metastases express receptors exceeds 80%. However, due to lack of ER+ models, little is known about the role of hormones in breast cancer spread or the effects of the LN microenvironment on hormone responsiveness. We developed metastasis models using ZsGreen labeled MCF-7 and T47D human breast cancer cells. Tumors were tracked in living mice by whole-body imaging, and macrometastases or micrometastases were detected by intravital imaging or fluorescence microscopy. Tumor growth was estrogen dependent and required for intratumoral lymphangiogenesis. Seventy-five percent of xenograft tumors generated LN metastases. Progesterone had minimal effects on tumor growth and metastasis. Occasionally more distant metastases were also observed. "Triads" of primary tumors, tumor filled draining lymphatic vessels, and tumor-filled LNs from the same mouse show that (a) proliferation, as measured by BrdU, is higher in the LN than in the primary tumor. (b) High ER levels are extensively down-regulated by estradiol in primary tumors. However, there is partial failure of ER down-regulation in LNs associated with (c) reduced PR expression. This suggests that ER are dysfunctional in the LN microenvironment and perhaps hormone resistant. (d) CD44 is sparsely expressed in primary tumor cells but homogeneously overexpressed in cells transiting the lymphatics and populating LNs. We hypothesize that CD44 expression targets tumor cells for transport to and growth in LNs. To determine if the LN microenvironment alters estrogen-dependent gene expression, we developed a unique model to identify estradiol-regulated genes in ER+ breast tumors and LN metastases. Fluorescent ER+ MCF-7 tumors were grown in ovariectomized nude mice supplemented with estradiol. Once axillary LN metastasis arose, estradiol was withdrawn (EWD), for 1 or 4 weeks, or continued, to assess estradiol responsiveness. On EWD, proliferation rates fell similarly in tumors and LN metastases. However, estradiol-dependent ER down-regulation and PR induction were deficient in LN metastases, indicating that ER transcriptional activity was altered in the LN. Cancer cells from estradiol treated and EWD primary tumors and matched LN metastases were isolated by laser capture microdissection. Global gene expression profiling identified transcripts that were regulated by the tissue microenvironment, by hormones, or by both. Interestingly, numerous genes that were estradiol regulated in tumors lost estradiol sensitivity or were regulated in the opposite direction by estradiol in LN metastases. We propose that the LN microenvironment alters estradiol signaling and may contribute to local antiestrogen resistance.

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P62-4: 27-HYDROXYCHOLESTEROL IS AN ENDOGENOUS SELECTIVE ESTROGEN RECEPTOR MODULATOR (SERM)

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Selective estrogen receptor modulators (SERMs) are estrogen receptor (ER) ligands whose relative agonist/antagonist activities vary in a cell- and promoter dependent manner. The molecular basis underlying this selectivity can be attributed to the ability of these ligands to induce distinct alterations in ER structure leading to differential

recruitment of coactivators and corepressors. Whether SERM activity is restricted to synthetic ligands or if molecules exist *in vivo* that function in an analogous manner remains unsolved. However, the recent observation that oxysterols bind ER and antagonize the actions of 17 β -estradiol (E2) on the vascular wall suggests that this class of ligands possesses SERM activity. We demonstrate here that 27-hydroxycholesterol (27HC), the most prevalent oxysterol in circulation, functions as a SERM, the efficacy of which varies when assessed with different endpoints. Importantly, 27HC positively regulates both gene transcription and cell proliferation in cellular models of breast cancer. Using combinatorial peptide phage display, we have determined that 27HC induces a unique conformational change in both ER α and ER β , distinguishing it from E2 and other SERMs. Thus, as with other ER ligands, it appears that the unique pharmacological activity of 27HC relates to its ability to impact ER structure and modulate cofactor recruitment. Cumulatively these data indicate that 27HC is an endogenous SERM with partial agonist activity in breast cancer cells and suggest that it may influence the pathology of breast cancer. Moreover, given the product-precursor relationship between 27HC and cholesterol, our findings have implications with respect to breast cancer risk in obese/hypercholesteremic individuals.

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P62-5: ESTROGEN RECEPTOR ALPHA PROTEIN COMPLEXES IN MCF-7 BREAST CANCER CELLS

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Background: Estrogen plays a major role in breast cancer, and therefore, ER is a major molecular target for therapeutics. Proteins in endogenous ER complexes may also be potential therapeutic targets. One of the most rapid events upon ER occupation with estrogen in the cell is ER tight nuclear binding. ER has been shown to interact with a large number of nuclear components and some of these associations probably play a role in tight nuclear binding. In this study we examine the molecular basis for estrogen induced tight nuclear binding of ER. We also attempted to purify and characterize endogenous ER protein complexes.

Methodologies: We combined the use of polyanions and ER mutant proteins to demonstrate the requirement for the DNA binding domain (DBD) of ER in estrogen-induced tight nuclear binding. We utilized protein crosslinkers to stabilize ER protein complexes for subsequent purification and attempts to identify components by mass spectrophotometry.

Results: We demonstrated that tight nuclear binding of ER required ER:DNA interactions. Hormone bound ER was extracted from the nucleus in low salt buffer using various polyanions, which mimic the phosphate backbone of DNA. The importance of specific ER:DNA interactions in mediating tight nuclear binding was supported by the 380-fold lower concentration of ERE-DNA necessary to extract estrogen occupied ER compared to polyanions. We demonstrated that tight nuclear binding required both the nuclear localization domain and the DBD of ER. We further demonstrated that ER:AIB1 interaction was not required for estrogen-induced tight nuclear binding. We captured endogenous protein complexes by treating whole cells with a cleavable protein-protein crosslinker (DTME) to maintain endogenous complexes followed by extraction of ER using high salt. We utilized western blot and sucrose gradient to confirm that ER was crosslinked into large complexes. We used a 3-step purification scheme that included: (1) extraction of crosslinked ER from isolated nuclei, (2) anti-ER α IgG Sepharose immunoaffinity column, and (3) ER immunoprecipitation. The resulting estrogen-occupied ER α -protein complex contained 5 proteins including ER α based on silver stain and western blot analysis. We attempted to identify the bands using tandem MS/MS mass spectrometry, but the final purification product did not contain a sufficient amount of ER or its potential binding partners for mass spectrometry analysis. Recovery of total ER in the pure complex was less than 1%.

Conclusions/Impact on Breast Cancer Research: Taken together, we propose a model in which tight nuclear binding of the estrogen-occupied ER is predominantly mediated by ER:DNA interactions. The effects of estrogen binding on altering DNA binding in whole cells is proposed to occur through estrogen-induced changes in ER:chaperone protein interactions, which alter DNA accessibility of ER but do not directly change ER affinity for DNA, which is similar for both unoccupied and occupied ER. We also conclude that occupied ER is not in a single, dominant complex, therefore the targeting of one binding partner may have limited utility for breast cancer therapeutics.

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P62-6: PHOSPHORYLATION-DEPENDENT ANTAGONISM OF SUMOYLATION DE-REPRESSSES PROGESTERONE RECEPTOR ACTION IN BREAST CANCER CELLS

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Progesterone receptors (PR) mediate proliferation during breast development and contribute to breast cancer progression in part by synergizing with peptide growth factors. We previously identified PR Ser294 as a hot spot for direct regulation of PR location, activity, and turnover in response to phosphorylation events. Herein, we sought to better understand how hormonal cross-talk alters PR function. We demonstrate that progestins (R5020 and RU486) induce rapid (15 min) sumoylation of PR Lys388; sumoylation represses PR transcriptional activity on selected PRE-driven and endogenous promoters and retards ligand-induced PR downregulation, phenotypes of Ser294 phospho-mutant (S294A) PRs. Consistent with this finding, we show that stabilized but weakly active S294A PR are heavily sumoylated. Conversely, desumoylated PRs, created by mutation of PR Lys388 (K388R) or by overexpression of SENP1 desumoylating enzymes, are hypersensitive to low progestin concentrations. Combination of K388R and S294A mutations (KRSA double-mutant PR) rescues both transcription and turnover of impaired phospho-mutant (S294A) receptors. Notably, phosphorylation events antagonize PR-B but not PR-A sumoylation. Treatment of cells with EGF or transient expression of activated MAP/ERK kinase, kinase (MEK), or CDK2 induces PR-B Ser294 phosphorylation and blocks PR-B sumoylation, thereby derepressing receptor activity; PR-A is resistant to these events. Modulation of reversible PR sumoylation in response to diverse hormonal signals provides a mechanism for rapid isoform-specific changes in hormone responsiveness. In the context of high protein kinase activities, such as during mammary gland development or breast cancer progression, phosphorylated PR-B are predicted to be undersumoylated, transcriptionally hyperactive, and unstable/undetectable.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0257.

P62-7: QUANTIFYING ER FUNCTION USING HIGH-THROUGH-PUT IMAGING IN BREAST AND OTHER CANCER CELLS

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A combined overstimulation of estrogen receptor- α (ER) and the EGF receptor (EGFR) may provide a strong stimulus for breast tumor growth and contribute to the resistance of tumor cells to antagonist therapy. The molecular mechanism of this activation remains poorly understood and continues to be an intense area of study. Our PRL-HeLa cell line is a novel tool developed for quantitative studies of ER function at the single-cell level (live and fixed). It is also highly amenable for use in new screening technologies involving high-throughput microscopy (HTM). Using this HTM-based approach, we are testing the hypothesis that PRL-array-containing cell lines can effectively be used to identify novel molecular players in ER α transcriptional activation and repression and will determine temporal patterns of function of all known ER α -coregulators. This systems-biology-level approach will integrate functional data from multiple readouts at a single cell level for ER α and CoRs including (1) nuclear targeting, (2) promoter array occupancy, (3) large-scale chromatin modeling, (4) histone modifications, and (5) mRNA synthesis.

The aims of our project are: (1) To document the pattern of ER α -CoR co-association with and dissociation from the ER α -targeted PRL-array in agonist-mediated activation (E2, EGF) or antagonist-mediated (Tam, ICI) repression of reporter transcription in PRL-HeLa cells using our panel of over 60 affinity purified antisera to known ER CoRs identified by proteomics. (2) To generate new chromosomally integrated PRL-arrays in ER-negative MCF7-C4/C412 breast cancer cells and to perform the assays proposed in Aim 1. (3) To conduct a screen for novel ER α coregulators using an extensive RNAi library and PRL-HeLa and PRL-MCF7-C4/C412 array-containing cells.

Construction of new reporter genes consisting of the PS2 promoter driving the expression of pmCherry has been completed. mCherry in these constructs is tagged with either a C-terminal or an N-terminal NLS for partitioning it to the nucleus. HA tags located in the C-terminus of each will provide for immunoassays. Reporters have been sequence verified and are currently being tested for estrogen-responsive expression and nuclear localization. Custom software development has continued to be able to quantify ER or CoR signals at the arrays; we are currently testing new multiplex routines that measure NR/CoR translocations, reporter gene activity, and cell cycle position. These routines will be run simultaneously in the promoter occupancy measurements using array-containing cell lines.

Data obtained from this project will be crucial to understanding the temporal ER responses to two physiological and breast cancer-related agonists and antagonists and will point the way toward development of new therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0605.

P62-8: MICRORNA SIGNATURES OF ER α : miR-221 AND miR-222 NEGATIVELY REGULATE ER α AND ASSOCIATE WITH TAMOXIFEN SENSITIVITY IN BREAST CANCER

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MicroRNAs (miRNAs) represent a novel class of genes that function as negative regulators of protein-coding gene expression. The loss of ER α expression in patients with breast cancer will not respond to endocrine therapy accompanying with poor prognosis. In addition to one third of breast cancers lacking ER α at the time of diagnosis, a fraction of breast cancers that are initially ER α -positive lose ER α expression during tumor progression. While ER α absence is a result of promoter hypermethylation in a subset of tumors, the molecular mechanism of ER α -negative and the molecule(s) involving ER α -hypermethylation are still largely unknown. Here, we performed whole genome miRNAs expression microarray in ER α -positive and -negative breast cell lines and primary tumor specimens. A distinct expression of a panel of miRNAs was detected between ER α -positive and -negative cells. Based on the ratio of ER α -negative/-positive, 11 miRNAs (≥ 1.5) are elevated and 10 (≤ 0.5) are downregulated. Further, we demonstrated that those elevated miRNAs in ER α -negative cells miR-221 and miR-222 directly target to 3'UTR of ER α . Ectopic expression of miR-221 and miR-222 in MCF7 cells resulted in decrease expression of ER α at protein and mRNA levels, whereas knockdown of miR-221 and miR-222 partially restored ER α expression in MDA-MB-468 cells. Notably, miR-221 and/or miR-222-overexpressing MCF7 became resistance to tamoxifen as compared to Blockit vector-transfected MCF7 cells and the knockdown of these 2 miRNAs rendered the MDA-MB-468 cells sensitive tamoxifen-induced cell growth arrest. These findings indicate that miR-221 and miR-222 play an important role in regulation of ER α and could be potential targets for restoring ER α expression and response to anti-estrogen therapy in breast cancer.

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P62-9: ERR γ : DOES AN ORPHAN NUCLEAR RECEPTOR LINK STEROID HORMONE BIOGENESIS TO ENDOCRINE RESISTANCE?

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Estrogen-related receptor gamma (ERR γ) is an orphan nuclear receptor with structural similarities to ER α and ER β . In addition to its ability to transactivate classical and imperfect estrogen response elements (EREs), ERR γ is a potent activator of transcription from steroidogenic factor-1 (SF-1) response elements (SF-IREs). Many genes regulated by SF-IREs control key aspects of cholesterol and fatty acid synthesis, important not only for generation of the plasma membrane but also for the synthesis of steroid hormones. In this study, we have investigated whether ERR γ expression and/or activity regulates the level of cholesterol in a pair of breast cancer cell lines—one sensitive to endocrine therapy (SUM44) and the other resistant to endocrine therapy (LCCTam, TAM).

We found that endocrine-resistant LCCTam (TAM) cells, which overexpress the orphan nuclear receptor ERR γ , contain significantly greater levels of cholesterol than endocrine-sensitive parental SUM44 breast cancer cells, and that siRNA-mediated knockdown of ERR γ in the resistant TAM cell line significantly reduces cholesterol content. SF-IRE activity is 3-fold higher in TAM cells as compared to SUM44 cells, while expression of an endogenous gene (HMGCS2) that contains a consensus SF-IRE is also significantly overexpressed in TAM cells relative to SUM44 cells. Together, these findings represent an important enhancement in our knowledge of breast cancer biology and potential therapeutic response.

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P62-10: DEFINING REGIONS OF THE ESTROGEN RECEPTOR- α THAT SELECTIVELY CONTROL ITS ESTRADIOL- AND TAMOXIFEN-STIMULATED ACTIVITY

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Estrogen drives the proliferation and growth of breast cancers by binding to the estrogen receptor-alpha (ER α) and stimulating transcription activation. Tamoxifen, which is used to prevent and treat breast cancer, blocks tumor growth by blocking estradiol's actions in breast, yet in other tissues tamoxifen can stimulate transcription activation. The F domain is a 42-amino acid region at the extreme carboxy-terminus of the estrogen receptor- α that, when deleted, eliminates the stimulatory activity of tamoxifen yet retains stimulatory activity of estradiol. The goal of this research was to identify the portions of the F domain responsible for this activity and to understand the mechanism(s) through which the F domain modulates the ligand-selective activity of the receptor.

Using the ability of a series of serial truncation mutants to stimulate transcription on an ERE-driven luciferase reporter, we found that deleting the last 16 residues of the ER α reduced the activity of the ER in response to both estradiol and tamoxifen and reduced its sensitivity to overexpression of the coactivator steroid receptor coactivator-1. When clones of MDA-MB-231 cells stably expressing the wild-type or truncated ER α were used, deleting the last 16 residues of the ER α reduced the ability of estradiol to stimulate transcription of the pS2 gene, and reduced the accumulation of the ER α at the pS2 promoter. By contrast, deletion of the entire F domain was required to eliminate tamoxifen's agonist activity on an ERE-driven promoter. These results show that portions of the ER α F domain selectively modulate the activity of the receptor.

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P62-11: THE ANDROGEN RECEPTOR INHIBITS ESTROGEN RECEPTOR α (ER) SIGNALING AND PREDICTS OUTCOME IN ER POSITIVE BREAST CANCERS

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Androgens inhibit the proliferation of breast cancer cells and this inhibitory effect appears to be dependent on the intracellular mediator of androgen action, the androgen receptor (AR). In a cohort of 219 invasive breast cancers, we have shown that positive AR immunostaining (median % positive cells $>$ or equal to 75) is significantly associated with estrogen receptor alpha (ER α) (χ^2 ; P value < 0.0001) and progesterone receptor (PR) (χ^2 ; P value = 0.001) status, and also a decreased risk of relapse and increased overall survival. Moreover, in ER α positive patients, AR positive immunostaining was independently associated with relapse-free survival and overall survival. Consistent with this, low AR immunostaining ($< 75\%$ positive cells) in the ER α positive cohort was associated with a 2.3 fold increased risk of relapse (P value = 0.024) and a 4.8 fold increased risk of cancer-related death (P value = 0.002). To investigate the functional significance of AR expression in ER α positive breast cancer cells, we examined the effect of AR on ER α signaling. Ligand activated full-length AR and a truncated, constitutively active AR (AR-T707) significantly inhibited the activity of ectopically expressed ER α in MDA-MB-231 breast cancer cells (ER α -negative and AR-negative), and endogenous ER α in T-47D breast cancer cells (ER α -positive and AR-positive). Adenoviral mediated expression of the AR-T707 resulted in complete abrogation of estradiol induced T-47D breast cancer cell proliferation. Deletion and mutation analyses indicated that the AR-DBD is both necessary and sufficient for inhibition of ER α activity. Consistent with this finding, mobility shift assays demonstrated that the full-length AR is able to bind to a consensus estrogen response element (ERE); however, no heterodimer formation between AR and ER α on an ERE was evident. Our findings suggest that the ability of the AR to inhibit ER α activity occurs either via formation of non-functional heterodimers between AR and ER α or by AR homodimers competing effectively for binding of ER α homodimers to EREs. Collectively the results of these studies further support the hypothesis that the equilibrium between ER α and AR is a critical determinant of breast cancer growth. Further studies are under way to investigate whether perturbations to this balance in the normal breast may contribute to increased breast cancer risk.

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P62-12: LACK OF A MAJOR PHENOTYPE IN SCAFFOLD ATTACHMENT FACTOR B2 (SAFB2)-NULL MICE REVEALS ALTERNATE FUNCTIONS IN SAFB2 COMPARED TO ITS PARALOG SAFB1

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Scaffold attachment factor B2 belongs to SAFB family. There are two members in the family, SAFB1 and SAFB2. Both SAFB1 and SAFB2 are multifunctional proteins that can bind both DNA and RNA and are involved in RNA processing and stress response. In addition, both B1 and B2 contain a transcriptional repression domain and can bind certain hormone receptors and repress their activity. We have generated SAFB1 knockout mice that show growth retard (due to low serum IGF1 level), male infertile, and dramatic defect in the development and function of reproductive system. To assess the role of SAFB2 and compare the functional difference between SAFB1 and SAFB2, we generated SAFB2 mutant mice through targeted deletion in embryonic stem cells. Unlike SAFB1 knockout mouse, SAFB2 knockout mice show normal in survival rate, fertility, and growth rate with normal serum IGF1 level. Tissue analysis by H.E. staining demonstrates there is no significant change in SAFB2 knockout mouse. Previous data revealed that the different expression patterns of SAFB1 and SAFB2 are found only in testis. While SAFB1 is highly expressed in germ cells, SAFB2 is highly

expressed in Sertoli cells in testis. In agreement with these results, SAFB2 knockout mice have a significant bigger testis than wild type. Immunohistochemistry staining of WT1 (mark of Sertoli cells) reveals that the Sertoli cells number is dramatic increased in SAFB1 knockout mice. Based on these results, we concluded that SAFB1 and SAFB2 have different function in development, growth, and reproduction.

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P62-13: METASTATIC TUMOR ANTIGEN 3 IS A DIRECT COREPRESSOR OF THE Wnt4 PATHWAY AND REGULATES MAMMARY DUCTAL BRANCHING

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Here we show that expression of MTA3 inhibits ductal branching in virgin and pregnant murine transgenic mammary glands. MTA3 also suppresses the Wnt4 pathway and, thus, these findings parallel phenotypic changes in *Wnt4*-null mice. MTA3 represses *Wnt4* transcription and Wnt4 secretion, inhibiting Wnt-target genes in mammary epithelial cells. Accordingly, knockdown of endogenous MTA3 stimulates Wnt4 expression and Wnt cellular targets. The MTA3–NuRD (nucleosome remodeling and deacetylase) complex physically interacts with the *Wnt4* chromatin in an HDAC-dependent manner, leading to suppression of the *Wnt4* gene and Wnt4-dependent morphogenesis. These findings identify MTA3 as an upstream physiologic repressor of *Wnt4* in mammary epithelial cells.

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P62-14: ACTIVATION OF THE STEROID AND XENOBIOTIC RECEPTOR, SXR, INDUCES APOPTOSIS IN BREAST CANCER CELLS

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The steroid and xenobiotic receptor, SXR, is a broad-specificity orphan nuclear receptor that regulates metabolism of diverse xeno- and endobiotic compounds. SXR is expressed at high levels in the liver and intestine and at lower levels in other tissues, including breast where its function was unknown. Here we describe a novel role for SXR as an inhibitor of breast cancer cell growth. The decreased breast cancer growth in response to SXR activation is associated with stabilization of p53 and up-regulation of cell cycle regulatory and pro-apoptotic genes such as p21, PUMA, and BAX. These gene expression changes are preceded by an increase in inducible nitric oxide synthase (iNOS) and nitric oxide (NO) in these cells. Inhibition of NO synthase blocked the induction of p53, demonstrating the requirement of NO for p53 induction in this system. The SXR activator-induced increase in iNOS was also inhibited by siRNA-mediated silencing of SXR, indicating that SXR activation both precedes and is necessary for subsequent regulation of iNOS expression. Transient transfection of a constitutively active form of SXR inhibits proliferation of MCF-7 cells, further emphasizing the role of SXR in blocking the growth of these cells. We conclude that SXR inhibits breast cancer growth and that this effect is mechanistically dependent upon the local production of NO and NO-dependent up-regulation of p53. These findings reveal a novel biological function for SXR and suggest that a subset of SXR activators may function as effective therapeutic and chemo-preventative agents for certain types of breast cancers.

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P62-15: TRANSCRIPTIONAL CONTROL OF STEROIDOGENIC ENZYMES BY ESTROGEN-RELATED RECEPTOR α AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA COACTIVATOR-1 α

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Estrogen is a well-characterized mitogen for many breast cancers, and increasing studies have indicated that extra-gonadal production of estrogen can be a biologically significant source of this mitogen. In fact, aromatase or Cyp19, the enzyme that converts androgens to estrogens, is expressed in many breast tumors and is thought to contribute to continued local production of estrogen in women after menopause or hysterectomy. Estrogen-related receptor alpha (ERR α) is an orphan nuclear receptor closely related to estrogen receptor whose expression in tumors correlates with unfavorable biomarkers and poor prognosis in breast cancer patients; however, its functional role in breast cancer is not known. We have found that ERR α can regulate expression of several steroidogenic enzymes, including Cyp19, Cyp11A1, Cyp17A1, and StAR, suggesting that it

may contribute to local production of steroids. A primary coactivator of ERR α is Peroxisome Proliferator Activated Receptor Gamma Coactivator-1 alpha (PGC-1 α), which is capable of coactivating ERR α to induce gene expression of each of these steroidogenic enzymes. Cyp11A1 and Cyp17A1 are known to catalyze the first two reactions in the steroidogenic pathway, converting cholesterol to pregnenolone, 17 α -hydroxypregnenolone and dehydroepiandrosterone (DHEA), and induction of Cyp11A1 and Cyp17A1 by PGC-1 α and ERR α produces functional enzymes that can synthesize DHEA from cholesterol precursors. Additionally, examination of the promoter regions of the Cyp17A1 promoter has yielded a fragment that demonstrates ERR α responsiveness. Taken together, these studies suggest a mechanism by which ERR α could contribute to breast cancer progression through transcriptional regulation of steroidogenic enzymes.

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P62-16: IDENTIFICATION OF UBIQUITIN SYSTEM COMPONENTS INVOLVED IN LIGAND-DEPENDENT TURNOVER OF ESTROGEN RECEPTOR

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Breast cancer is the most commonly diagnosed cancer in women in the United States. Estrogen receptor-alpha (ER α), a member of the nuclear receptor superfamily of ligand-inducible transcription factors, plays an important role in breast cancer biology and therapy. Fulvestrant (Faslodex, ICI 182,780), an antagonist of ER α , is currently being used in the treatment of advanced breast cancer. Fulvestrant induces rapid turnover of ER α . However, the precise mechanism underlying this process and its contribution to the therapeutic action of fulvestrant are not understood.

This proposal aims to identify and evaluate the importance of the ubiquitin proteasome system (UPS) components that are involved in the fulvestrant-mediated turnover of ER α . The specific aims of the proposal are as follows:

1. To identify the components of the UPS that are responsible for fulvestrant-mediated turnover of ER α .
2. To understand the biochemical mechanism by which fulvestrant activates turnover of ER α .
3. To evaluate whether the ubiquitin ligase involved in fulvestrant-mediated turnover of ER α is required for the therapeutic effect of fulvestrant in a xenograft mouse model of breast cancer.

In the first specific aim, a mass-spectrometry and a short hairpin RNA-based approach will be used to identify the UPS components that are responsible for fulvestrant-mediated turnover of ER α . The second specific aim will bring out the difference(s), if any, between the natural ligand-stimulated and the fulvestrant-mediated turnover of ER α . Also, it will involve reconstitution of the fulvestrant-dependent turnover pathway in vitro with purified components. The third specific aim will clarify the role of UPS pathway for the tumor growth inhibitory role of fulvestrant in vivo.

The line of research proposed here has implications for both basic biology and clinical medicine. The project will lead to a greater understanding of the biology behind ER α turnover in general as well as in the specific context of fulvestrant-mediated turnover. The expression level of the ligase (that is responsible for fulvestrant-mediated turnover of ER α) could turn out to be a useful parameter in predicting the clinical efficacy of the drug. Moreover, understanding the mechanism of action of fulvestrant may provide clues to overcome drug resistance that is common in any kind of targeted therapy. Also, this knowledge could be applied in the future to create novel drugs that induce proteasome-mediated degradation of other clinically relevant targets.

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P62-17: AIB1/SRC-3 IS REQUIRED FOR NEU (ERBB2/HER2) ACTIVATION, SIGNALING, AND MAMMARY TUMORIGENESIS IN MICE

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Overexpression of the oncogene amplified in breast cancer 1 (AIB1/SRC-3) induces mammary tumorigenesis in mice. In breast cancer, high levels of AIB1/SRC-3 and the growth factor receptor HER2/*neu* predict resistance to endocrine therapy and poor outcome. However, a mechanistic relationship between AIB1/SRC-3 and HER2/*neu* in the development of breast cancer has not been demonstrated. Here we show that deletion of one allele of *SRC-3* significantly delays *Neu*-induced mammary tumor development in mice. Homozygous deletion of *SRC-3* in mice completely prevents *Neu*-induced tumor formation. By 3–4 months of age, *Neu*/SRC-3 mice exhibit a noticeable reduction in lateral side bud formation accompanied by reduced cellular levels of phosphorylated *Neu* compared to *Neu*/SRC-3^{wt} mice. In *Neu*-induced tumors, high levels of

SRC-3, phospho-*Neu*, cyclin D1, cyclin E, and PCNA expression are observed accompanied by activation of the AKT and JNK signaling pathways. In comparison, phospho-*Neu*, cyclin D1, and cyclin E are significantly decreased in *Neu*/SRC-3 tumors, proliferation is reduced, and AKT and JNK activation is barely detectable. Our data indicate that AIB1/SRC-3 is required for HER2/*neu* oncogenic activity and for the phosphorylation and activation of the HER2/*neu* receptor. We predict that reducing AIB1/SRC-3 levels or activity in the mammary epithelium could potentiate therapies aimed at inhibiting HER2/*neu* signaling in breast cancer.

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P62-18: ELEVATED LEVELS OF CIRCULATING IGF-I DO NOT MODULATE ErbB2-INDUCED MAMMARY TUMORIGENESIS

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Epidemiological evidence indicates that elevated circulating levels of insulin-like growth factor-I (IGF-I) predict breast cancer risk in premenopausal women. While abundant evidence exists for autocrine/paracrine action of IGF-I in breast cancer, little is known about the role of circulating IGF-I. Furthermore, most experimental models with lowered IGF-I levels have delayed or inhibited mammary tumorigenesis, with few models directly confirming the association between moderately high systemic IGF-I levels and mammary tumorigenesis. Therefore, we assessed the ability of circulating IGF-I to initiate and/or progress mammary tumorigenesis using the newly developed

TTR-IGF-I liver transgenic mouse model; the first mouse model that overexpresses IGF-I in the liver resulting in increased systemic circulating levels of IGF-I. We crossed heterozygous TTR-IGF-I mice with heterozygous MMTV-ErbB2 mice to generate 4 different genotypes: TTR-IGF-I/MMTV-ErbB2 (bigenic), TTR-IGF-I only, MMTV-ErbB2 only, and wild type (wt). TTR-IGF-I and bigenic mice showed a 35% increase in circulating total IGF-I compared to ErbB2 and FVB wt control mice. TTR-IGF-I transgenic mice weighed more than wt or MMTV-ErbB2 mice, although the difference was small (~10%) and was only apparent from 6-12 weeks of age. Elevation of systemic IGF-I had no effect upon ErbB2-induced mammary tumorigenesis, with median time to tumor formation being 30 wks and 33 wks in bigenic and ErbB2 mice, respectively ($p=0.65$). Furthermore, both groups of mice had the same number of tumors (ErbB2 – 2.2 tumors/mouse and bigenic 2.3 tumors per mouse). A preliminary analysis indicated that bigenic mice did show an increased number of lung macro-metastases (5 in bigenic versus 2 in ErbB2). We measured the rate of tumor growth and found that increased levels of IGF-I had no effect upon ErbB2 initiated tumors ($p=0.23$). Finally, elevated IGF-I alone was insufficient (alone) to cause mammary gland tumors. Whole mount and H&E analysis revealed no major morphological differences in ductal branching and tumor type (solid adenocarcinomas) between bigenic and ErbB2 mammary glands. Immunoblot analysis revealed that circulating levels of IGF-I had no effect on mammary gland signaling in that phospho-IGF-I receptor and phospho-AKT was similarly activated in both the bigenic and ErbB2 only tumors compared to their respective normal mammary glands. Using the first transgenic animal model to simulate circulating levels of IGF-I that may be comparable to levels in premenopausal women susceptible to breast cancer, we have shown that moderately high levels of systemic IGF-I have no effect on ErbB2-induced tumorigenesis.

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ENDOCRINE PATHOGENESIS II

Poster Session P63

P63-1: HORMONES OF PREGNANCY, AFP, AND REDUCTION OF BREAST CANCER RISK

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Background: Parity profoundly reduces women's risk of acquiring breast cancer later in life. It has been reasoned that hormones (either estradiol E₂ or estrion E₃), progesterone (P), or human chorionic gonadotropin (hCG) in the serum of pregnant women might lead to that reduction in risk. These agents have been shown to reduce breast cancer incidence in carcinogen-treated virgin rats. We investigated the hypothesis that exogenously added E₂, E₃, P, or hCG are not the proximal effectors of risk reduction, but that they elicit α -fetoprotein (AFP) from the non-pregnant liver and that AFP is the proximal agent by which reduction of breast cancer risk is obtained.

Methods: Methylnitrosourea (MNU)-exposed animals were treated with saline, E₃, E₂ + P, E₃ + P, hCG, or were allowed to experience pregnancy, and AFP levels were measured in the serum and subsequent tumor incidence was recorded. Human HepG2 liver cells in culture were treated with E₃, E₂ + P, or hCG and elicited AFP was measured in the media. The HepG2 culture media containing elicited AFP was assessed for its ability to inhibit proliferation of T47D cells when applied to these human breast cancer cells in culture, and to inhibit the estrogen-induced phosphorylation of the estrogen receptor in T47D cells.

Results: For each condition in the prevention studies, hormone treatment reduced the incidence of breast cancer to an extent similar to that reported by the original studies. In each condition, AFP levels in serum were elevated over that in control animals. In culture, treatment of human liver cells with E₃, E₂ + P, or hCG, but not P alone, led to increased levels of AFP in the media. Media containing hCG-elicited AFP inhibited the estrogen-stimulated proliferation of T47D cells in culture, and inhibited phosphorylation of the estrogen receptor, whereas, estrogens and hCG did not inhibit the growth of these tumor cells in culture.

Conclusion: Since the hormones of pregnancy elicit AFP from the liver, and AFP but not the hormones of pregnancy has direct anti tumor properties, it is concluded that AFP is the proximal agent through which reduction in breast cancer incidence is realized from the experience of pregnancy.

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P63-2: CONTROL OF ESTROGEN-REGULATED MICRORNA EXPRESSION BY AKT

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Background and Objective: Estrogen induces or represses gene expression by acting as a ligand for estrogen receptor alpha (ER α) and ERbeta. To date, majority of mechanistic studies were focused on estrogen inducible gene expression and anti-estrogen based therapies were mostly designed and assessed for their ability to inhibit estrogen-induced gene expression. However, recent studies show that ~70% of genes regulated by estrogen are repressed in breast cancer cells suggesting that estrogen-repressible genes play a significant role in breast cancer. Such high percentage of repression is achievable if estrogen modulates a gene regulatory system other than classical transcription initiation machinery. The objective of this study was to investigate whether estrogen regulates gene expression through microRNAs and whether extracellular signal activated kinases such AKT modulate estrogen-regulated microRNA expression.

Methodologies: RNA from untreated and estrogen-treated (10-8 M, 3 hours) MCF-7 (pQXIP) and MCF-7 cells overexpressing constitutively active AKT (CA-AKT) were hybridized to miRNA-array comprising 248 human microRNAs. MicroRNA expression was further verified by qRT-PCR. The effect of estrogen-regulated microRNAs on target gene expression was determined by western blot analysis. Chromatin-immunoprecipitation coupled microarray was used to identify ER α binding sites in genomic region encoding microRNAs. Various bioinformatics tools were used to predict mechanisms of estrogen-regulated microRNA expression and to identify putative targets of microRNAs.

Results: Estrogen increased the expression of 21 microRNAs and decreased the expression of 7 microRNAs in pQXIP cells. AKT completely changed the pattern of microRNA expression as estrogen increased the expression of one and reduced the expression of 20 microRNAs in CA-AKT cells. Estrogen increased the expression of most of the Let-7 family members in pQXIP but not CA-AKT cells and consequently reduced the protein levels of Let-7 target Ras in pQXIP cells. Five of the estrogen-regulated microRNAs contain ER α binding sites while the remaining microRNAs appears to be regulated by transcription factors whose expression is under the control of estrogen and/or AKT. MicroRNA upregulated by estrogen may target ~486 (326+160) whereas microRNAs downregulated by estrogen may target ~551 (348+203) estrogen-

regulated genes; however, multiple microRNAs may target the same gene thus overall number of genes may be lower than predicted.

Conclusions: Estrogen controls gene expression at post-transcriptional level through microRNA; Ras is one of the main target. By enhancing the expression of Let-7 family, estrogen may promote differentiation of luminal epithelial cells, which is abrogated by extracellular signal activated kinases such as AKT.

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P63-3: HISTONE DEACETYLASE 2 (HDAC2) MEDIATES THE POTENTIATION OF TAMOXIFEN BY HDAC INHIBITORS

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Background: Histone deacetylases (HDACs) play a central role in many biological functions and may be involved in the pathogenesis of breast cancer. HDAC inhibitors have been shown to modulate estrogen receptor (ER) expression and may restore tamoxifen sensitivity to hormone-insensitive tumors. The interaction of HDAC inhibitors with tamoxifen suggests an important role of HDACs in ER signaling; however, very little is known as to which HDAC enzyme interacts with ER and which HDAC enzyme(s) is a relevant target for antihormonal therapy. We evaluated the roles of specific HDAC isoenzymes and their inhibition on both ER and progesterone receptor (PR) signaling and their importance in response to endocrine therapy.

Method: The roles of individual HDAC isoenzymes on ER and PR expression and their functions were evaluated by depletion of select HDAC enzymes using siRNA or pharmacological inhibition.

Results: Co-treatment of breast cancer cell lines with HDAC inhibitors and the anti-estrogen, tamoxifen, resulted in synergistic antitumor activity with simultaneous depletion of both ER and PR. Selective inhibition of HDAC2, but not HDAC1 or HDAC6, was sufficient to potentiate tamoxifen-induced apoptosis in ER/PR-positive cells. Depletion of HDAC1 and HDAC6 was associated with downregulation of ER but not PR. Only the selective depletion of HDAC2 downregulated both ER and PR expression and was sufficient to potentiate tamoxifen.

Conclusions: Selective depletion of HDAC2 resulted in simultaneous depletion of ER and PR, and potentiated the effects of antihormonal therapy in ER-positive cells. A more effective pharmacological inhibition of HDAC2 and evaluation of HDAC2 and PR as therapeutic targets or as predictive markers in hormonal therapy may be considered when combining HDAC inhibitors and hormonal therapy. The effect of HDAC enzymes is currently being evaluated in clinical samples.

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P63-4: PROLACTIN ANTAGONIZES CHEMOTHERAPEUTIC-INDUCED CYTOTOXICITY IN BREAST CANCER CELLS

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Background: Breast cancer patients are treated with various anti-cancer drugs, including the DNA damaging agent doxorubicin and the microtubule altering drugs taxol and vinblastine. However, resistance to chemotherapy is a major concern, with patients especially unresponsive to the DNA intercalating drug cisplatin. Prolactin (PRL) is a 23kd protein whose target is the breast, where it acts as a mitogen and survival factor. PRL is produced by both the pituitary and the breast, with expression of its receptors higher in tumors than in the normal breast. We previously reported that nude mice inoculated with PRL-overexpressing breast cancer cells developed faster growing tumors with increased expression of the anti-apoptotic protein Bcl-2.

Specific Aims: Given its action as a survival factor, we hypothesized that PRL antagonizes the cytotoxic effects of chemotherapeutic drugs in breast cancer. The specific aims were to: (1) characterize the sensitivity of several breast cancer cell lines to anti-cancer drugs and determine if PRL antagonizes their cytotoxicity, (2) examine if PRL opposes cisplatin-induced apoptosis, and (3) determine the mechanism by which PRL protects these cells from cisplatin cytotoxicity.

Results: Cisplatin, doxorubicin, vinblastine, and taxol induced dose-dependent decreases in cell viability that were completely or partially antagonized by low doses (25 ng/ml) of PRL. Such protection was observed using MDA-MB-468 (468) and MDA-MB-231 as well as T47D cells. The remainder of the experiments focused on the effects of cisplatin on 468 cells. To determine whether PRL antagonizes apoptosis, cells were labeled with Annexin-V/propidium iodide and analyzed by flow cytometry. Cisplatin-induced apoptosis was prevented upon pretreatment with PRL. As was also revealed by flow cytometry, cisplatin induced a G2/M cell cycle arrest. A combination of staining for phosphorylated histone H3 and Western blotting for cyclin B expression confirmed cell arrest at G2; this checkpoint was bypassed by PRL treatment. DNA damage was assessed by staining with γ -H2AX, which recognizes double-strand breaks.

The majority of the cells were positive for γ -H2AX following cisplatin treatment, but only minimal staining was seen when cells were pre-exposed to PRL. Ongoing studies are using ICP mass spectrometry to determine whether PRL blocks the transport of cisplatin to the nucleus. Additional experiments examine whether PRL alters apoptotic/anti-apoptotic Bcl-2 proteins.

Conclusions: This is the first demonstration that PRL, a natural hormone, confers a broad range chemoresistance in breast cancer cells. Of particular importance is the fact that protection by PRL is not limited to one cell line and extends to several anti-cancer drugs. The clinical implication is that circulating and/or locally produced PRL reduce the efficacy of chemotherapy in breast cancer patients. Understanding the mechanism by which PRL exerts its anti-cytotoxic actions is especially relevant to cisplatin, a widely used and highly effective chemotherapeutic agent that nonetheless has shown little success in the treatment of breast cancer. Future therapies aimed at reducing PRL levels or blocking its actions should improve the efficacy of chemotherapy and expand the available drug options.

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P63-5: AIB1 KNOCKOUT MICE ARE RESISTANT TO CHEMICAL CARCINOGEN-INDUCED MAMMARY TUMORIGENESIS

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AIB1 (SRC-3, p/CIP, RAC3, ACTR, TRAM-1, or NCoA-3) is a transcriptional coactivator for nuclear receptors and certain other transcription factors and is a newly defined oncogene overexpressed in human breast cancer. Although the role and molecular mechanism of AIB1 in normal physiology and in breast cancer are currently under intensive investigation, the role of AIB1 in determination of the susceptibility of mammary gland to chemical carcinogens remains uncharacterized. In this study, we used backcrossed FVB wild-type (WT) and AIB1 mutant mice to assess the role of AIB1 in mammary gland development and in carcinogen-induced tumorigenesis. We show that mammary ductal growth was delayed in AIB1^{-/-} mice with FVB strain background, and mammary ductal outgrowths emanating from the AIB1^{-/-} mammary epithelial transplants in WT mice also were attenuated, indicating that the role of AIB1 in mammary ductal growth is a mammary epithelial autonomous function. In mice treated with the chemical carcinogen 7,12-dimethylbenz [a] anthracene (DMBA), AIB1 deficiency protected the mammary gland, but not the skin, from tumorigenesis. AIB1 deficiency suppressed the upregulation of the insulin receptor substrate (IRS)-1 and IRS-2 and thereby inhibited the activation of Akt, the expression of cyclin D1, and cell proliferation. The suppression of these components for insulin-like growth factor-1 signaling might be partially responsible for the decreased DMBA-induced mammary tumor initiation and progression in AIB1^{-/-} mice. Our results suggest that AIB1 may serve as a potential target for prevention of carcinogen-induced breast cancer initiation and for treatment of breast cancer progression.

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P63-6: THE IMPACT OF STRESS ON TUMOR GROWTH: THE SIGNIFICANCE OF PERIPHERAL CORTICOTROPIN RELEASING FACTOR

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Background and Objectives: Corticotropin releasing factor (CRF) is the primary neuropeptide that mediates the hypothalamic response to stress. CRF has been detected not only in the central nervous system but also in peripheral tissues and organs. CRF is secreted from autonomic neurons in peripheral tissues upon stress affecting the surrounding cells, but it has also been detected in epithelial cells and immune cells. Even though systemic stress has been reported to affect cancer cell growth primarily due to suppression of the immune response, no information is available on the impact of peripheral CRF on breast cancer cell proliferation and metastasis. The aim of our study is to determine the impact of peripheral CRF on breast cancer growth and invasiveness and to propose a novel potential mechanism on the crosstalk between stress and tumor growth.

Methodology: We employed the mouse breast cancer cell line 4T1 to test the impact of CRF on breast cancer cell motility and invasiveness in culture and in vivo. In culture, cells were treated with CRF, and the expression of tumor-related cytokines and chemokines was determined by ELISA-based array, and expression of genes related to metastasis was determined by gene expression arrays. For the in vivo experiments, 4T1 breast cancer cells were transferred orthotopically in mice, and tumor growth was assessed in the presence or absence of CRF agonists and antagonists.

Results to Date: We first characterized the CRF receptors expressed on 4T1 cells and found that they express only CRF₁ receptor, the primary receptor for CRF. Treatment of 4T1 cells with CRF revealed that it had no effect on cell proliferation and apoptosis. We further tested the effect of CRF on several tumor-derived cytokines and chemokines using array technology and found that CRF significantly suppressed the expression of macrophage inflammatory protein-1 alpha (MIP-1alpha), a chemokine known to induce macrophage recruitment. Low levels of MIP-1alpha have been associated with impaired immune response against the tumor. We then used a boyden chamber invasiveness assay and found that CRF promoted cell invasiveness through extracellular matrix. To validate the effect of peripheral CRF on breast cancer cell growth, we transfected 4T1 cells with a plasmid expressing green fluorescent protein (GFP) and implanted them in the mammary fat pad of mice to monitor tumor growth in vivo using laser fluorescence tomography. Mice were treated with CRF receptor agonists and antagonists to determine tumor growth, angiogenesis, and invasiveness.

Conclusions: Our preliminary findings suggest that CRF has a direct effect on breast cancer cell invasiveness. The results of the study when completed will allow us to determine the significance of peripheral CRF on breast cancer and suggest novel therapeutic approaches using CRF receptor antagonists. Moreover, the results will provide a novel link between stress and tumor growth.

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P63-7: AUTOCRINE HUMAN GROWTH HORMONE REGULATES CYTOCHROME P450 AROMATASE IN A HUMAN MAMMARY CARCINOMA CELL LINE AND REDUCES SENSITIVITY TO AN AROMATASE INHIBITOR IN VITRO

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Breast cancer is now the leading female cancer in developed countries. Anti-estrogen therapies, such as selective estrogen receptor modifiers and aromatase inhibitors (AIs), are currently the frontline treatment for hormone-sensitive mammary tumours. However, development of resistance to these treatments is a major clinical challenge that has not yet been adequately addressed.

We have previously demonstrated the key role that autocrine human growth hormone (hGH) plays in oncogenic transformation and progression of mammary carcinoma, both in vitro and in vivo. Autocrine hGH enhances cell proliferation and survival in immortalized human mammary epithelial cells (HMECs) through differential gene regulation. In addition, expression of autocrine hGH is sufficient to promote oncogenic transformation of immortalized HMECs in vitro and promote tumor formation in vivo. Furthermore, autocrine hGH promotes migration, invasion, and epitheliomesenchymal transition in the mammary carcinoma cell line, MCF-7.

Here we demonstrate that autocrine hGH transcriptionally upregulates a key enzyme involved in estrogen biosynthesis, cytochrome P450 aromatase, in the human mammary carcinoma cell line, MCF-7. Semi-quantitative RT-PCR and a luciferase promoter assay utilizing the aromatase II/I.3 promoter demonstrated an upregulation of the aromatase gene in hGH producing cells when compared with a control cell line. A concomitant increase in aromatase protein levels was demonstrated by Western blot analysis. Autocrine hGH expression in MCF-7 cells reduced sensitivity to the AI, exemestane, when compared with a control cell line.

Our results demonstrate that forced expression of autocrine hGH in MCF-7 cells upregulates P450 aromatase expression and reduces sensitivity to the aromatase inhibitor Exemestane. Consequently, functional hGH antagonism may increase sensitivity to AIs and improve the prognosis of patients with hormone-sensitive breast cancer.

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P63-8: PROHIBITIN IS INVOLVED IN THE ANTIPROLIFERATIVE ACTION OF VITAMIN D IN BREAST CANCER CELLS

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Previous studies from our laboratory have shown that 1 α -Hydroxy-24-ethyl-cholecalciferol (1 α (OH)D5) inhibits chemically induced carcinogenesis in rats as well as exhibits antiproliferative properties for VDR positive breast cancer cells. In ER+ cells 1 α (OH)D5 downregulates estrogen receptor and estrogen inducible genes, whereas in VDR+, ER- cells it induces cell differentiation. Although it is established that the action of vitamin D is mediated by nuclear VDR, the genomic regulation of the sensitivity to vitamin D is not clear. The microarray analysis showed that transformation of MCF12F cells by MNU resulted in differential expression of 320 genes including prohibitin, which is involved in cell cycle regulation. We therefore proposed that pro-

hibitin could play a role in mammary carcinogenesis and in enhancing sensitivity to vitamin D. We first evaluated the effect of vitamin D5 on prohibitin expression. Real-time RT-PCR analysis demonstrated that 1 α (OH)D5 upregulated prohibitin in ER-positive breast cancer cells, western blot analysis confirmed that in ER-positive BT474 cells, vitamin D5 upregulated prohibitin protein expression, suggesting that prohibitin is a potential vitamin D target gene. By using the MCF-7 cells expressing tetracycline-inducible prohibitin-model (Tet-On model), we found that overexpression of prohibitin consistently inhibited cell proliferation and the inhibition was further enhanced by treating the cells with 1 α (OH)D5. Knock-down of prohibitin mRNA in MCF-7 cells using siRNA demonstrated higher proliferation rate and resistance to vitamin D treatment. The confocal microscopic analysis showed that prohibitin co-localized with VDR in the nuclei of MCF-7 cells, but we failed to detect the direct physical interaction between prohibitin and VDR from cell lysates. Real-time RT-PCR analysis of VDR target gene CYP24 also failed to demonstrate the direct interaction between prohibitin and VDR, since CYP24 transactivation by vitamin D was not consistently affected by prohibitin level. These results suggest that prohibitin is a potential vitamin D target gene and contributes to cellular response to vitamin D without direct physical interaction with VDR and that prohibitin may also serve as a potential target gene for breast cancer prevention and treatment with efficacious vitamin D analogs.

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P63-9: ANDROGEN REGULATES KITL EXPRESSION IN NORMAL BREAST EPITHELIUM AND AN ANDROGEN RECEPTOR POSITIVE BREAST CANCER CELL LINE

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Androgen receptor (AR) signaling may play an important role in maintaining homeostasis of breast tissue, in part by limiting the proliferative effects of estrogen on epithelial cells. Disruption of AR signaling could thereby promote development of breast cancer by allowing unopposed oestrogen activity. To test this hypothesis, it is critical to better understand AR signaling and identify key AR-regulated genes required for growth and development of normal breast tissue. We are pursuing this goal through use of an in vitro breast explant culture system, which maintains tissue morphology and sex steroid receptor expression, and thereby allows examination of steroid responses in a context that represents relatively normal epithelial and stromal architecture. Using this model, we examined expression and androgen regulation of kit ligand (KITL), recently identified as a candidate AR-regulated gene in ovarian tissue. KITL signals through its receptor c-kit, and both are expressed in normal breast epithelium. Reduction of KITL and c-kit expression has been observed with progression of breast cancer in a number of studies, suggesting that this cytokine signaling pathway forms an autocrine loop that is important to normal breast epithelial cell function. We performed immunohistochemistry for KITL on paraffin-embedded normal breast explant tissues at the following time-points: (1) day of collection; (2) following 48 hours of culture in steroid-free conditions; (3) following a subsequent 24 or 48 hours of culture in steroid-stripped media supplemented with the native androgenic ligand, 5 α -dihydrotestosterone (DHT; 1nM). As observed in other studies of normal breast tissue, expression of KITL protein was intense in breast epithelial cells of explant tissues on day of collection. This expression was markedly reduced following 48 hours of culture in steroid-free conditions but returned to normal levels following exposure to DHT at 24 and 48 hours (Figure 1). We also observed that KITL mRNA is expressed at low levels in the breast cancer cell line MDA-MB-453 (AR+; ER-; PR-), but is increased 5-fold after 10 hours of treatment with DHT (1 nM). Collectively, these results suggest that KITL is positively regulated by androgen signaling at the mRNA and protein levels in breast epithelial cells, and this up-regulation may be a means by which androgens regulate homeostasis in the breast.

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P63-10: ABNORMAL MAMMARY GLAND DEVELOPMENT AND GROWTH RETARDATION IN FEMALE MICE AND MCF7 BREAST CANCER CELLS LACKING ANDROGEN RECEPTOR

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Phenotype analysis of female mice lacking androgen receptor (AR) deficient (AR-/-) indicates that the development of mammary glands is retarded with reduced ductal

branching in the prepubertal stages, and fewer Cap cells in the terminal end buds, as well as decreased lobuloalveolar development in adult females, and fewer milk-producing alveoli in the lactating glands. The defective development of (AR-/-) mammary glands involves the defects of insulin-like growth factor I-insulin-like growth factor I receptor and mitogen-activated protein kinase (MAPK) signals as well as estrogen receptor (ER) activity. Similar growth retardation and defects in growth factor-mediated Ras/Raf/MAPK cascade and ER signaling are also found in (AR-/-)MCF7 breast cancer cells. The restoration assays show that AR NH2-terminal/DNA-binding domain, but not the ligand-binding domain, is essential for normal MAPK function in MCF7 cells, and an AR mutant (R608K), found in male breast cancer, is associated with the excessive activation of MAPK. Together, our data provide the first in vivo evidence showing that AR mediated MAPK and ER activation may play important roles for mammary gland development and MCF7 breast cancer cell proliferation.

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P63-11: BIDIRECTIONAL CROSSTALK BETWEEN LEPTIN AND IGF-1 SIGNALING TRANSACTIVATES EPIDERMAL GROWTH FACTOR RECEPTOR AND PROMOTES INVASION AND MIGRATION OF BREAST CANCER CELLS

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Recently, there has been considerable interest in the endocrine, paracrine and autocrine effects of adipocytokines in the development of breast cancer. Adipocytokine leptin, the key player in the regulation of energy balance and body weight control also acts as a growth factor on certain organs in both normal and disease states. We have previously shown that leptin induced growth stimulation of breast cancer cells by recruiting histone acetyltransferases and mediator complex to cyclin D1 promoter via activation of Stat3. Epidemiological studies have shown that increased level of IGF-1 in the circulation is associated with excessive proliferation and survival signals implicated in the development of breast cancer. Interestingly, we found bidirectional crosstalk between IGF-1 and leptin signaling as IGF-1 induced significant tyrosine phosphorylation of leptin receptor (Ob-Rb) and leptin induced tyrosine phosphorylation of IGF-1 Receptor (IGF-1R). Combined treatment of leptin and IGF-1 induced synergistic activation of Ob-Rb and IGF-1R along with signaling molecules of leptin signaling, Akt and ERK. Furthermore, IGF-1 and leptin synergistically transactivated epidermal growth factor receptor (EGFR) and induced proliferation. Intriguingly, we also found that leptin and IGF-1 potentially induced invasion of breast cancer cells in Matrigel invasion and ECIS invasion assays which was effectively reduced by pharmacological inhibitors of EGFR. Importantly, inhibition of EGFR activation also reduced leptin- and IGF-1-induced migration of breast cancer cells on fibronectin matrix. Taken together these data indicate a novel bidirectional crosstalk between leptin and IGF-1 signaling that transactivates EGFR and promotes invasiveness and migration of breast carcinoma cells.

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P63-12: PROGESTERONE RECEPTOR-MEDIATED REGULATION OF CELL CYCLE IN BREAST CANCER

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Progesterone is a naturally occurring steroid hormone that functions by binding to the progesterone receptor (PR) thereby enabling the receptor to bind DNA, recruit cofactors, and induce the transcription of target genes. In the breast and other tissues of the female reproductive system, progesterone plays an important role in normal development and function. However, recent data suggest that PR also contributes to the proliferation of breast cancer cells. Given that over half of all breast cancers express PR, there is an unmet potential for the use of PR modulators (PRMs) to inhibit breast tumor growth. Until the molecular mechanisms governing PR-mediated regulation of cell cycle are completely understood, however, it may be difficult to identify appropriate PRMs for clinical use. Recent data from our lab show that in T47D breast cancer cells, agonist-bound PR-B functions in a direct manner to induce transcription of E2F1 and E2F2, key regulators of cell cycle progression. In addition, we demonstrate that PRMs, which do not induce classic PR target genes, may activate E2F signaling and stimulate proliferation. We intend to dissect the molecular mechanisms behind this regulation and to further explore how E2F activity correlates with the proliferative response of various PRMs.

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P63-13: ESTRADIOL, IN MODERATE BUT NOT LOW DOSAGES, ADMINISTERED TO OVARECTOMIZED RATS HAS ANXIOLYTIC AND ANTI-DEPRESSANT-LIKE EFFECTS AND TROPHIC EFFECTS IN PERIPHERAL TISSUES

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Background: The ovarian steroid, estradiol (E_2), has a wide range of effects in the brain and body. In the brain, E_2 can influence aspects of psychological function, such as anxiety and/or mood. Indeed, some women may be particularly vulnerable to anxiety when their endogenous E_2 levels have declined, such as occurs premenstrually, postpartum, and/or after menopause. Results of clinical and basic animal research suggest that E_2 and/or other estrogenic compounds, may have beneficial effects for anxiety behavior. However, concerns about the utility and safety of E_2 -based therapies include potential risks associated with their trophic effects in reproductive tissues (mammary glands, uteri), and findings that some, but not all, women respond favorably to these therapies. As such, the relative psychological and trophic effects of E_2 and estrogenic compounds were investigated.

Research Design/Methods: To begin to investigate the relationship between E_2 's potential to have beneficial effects in the brain (on psychological processes) and negative proliferative effects in the body (on tumors, uterine weights), an animal model of "menopause" and E_2 -replacement was utilized. Young, adult Long-Evans rats were ovariectomized (ovx) to remove the main endogenous source of E_2 . One week after surgery, when E_2 levels are at nadir, rats were administered a low dosage of a chemical carcinogen (DMBA) or an inert control substance. One week later, and for 13 subsequent weeks, rats were administered a subcutaneous injection of placebo (oil vehicle), a low dosage of E_2 (0.03 mg/kg) or a moderate dosage of E_2 (0.09 mg/kg) and were tested in several tasks to assess anxiety-like (open field, elevated plus maze, light-dark transition) and depression-like (forced swim test) behavior. Behavior in control tasks that are typically altered by E_2 (sexual response, motor behavior) were assessed. At the end of the study, incidence, number, and size of tumors, uterine weight, and circulating E_2 levels were determined.

Summary of Results/Conclusion: The moderate concentration of E_2 (0.09 mg/kg) decreased anxiety-like behavior (greater entries in the center of the open field, more time spent in the light of the light-dark transition task) compared to that of rats dosed with placebo or the lower E_2 (0.03 mg/kg) concentration. A similar pattern was observed for 0.09 mg/kg E_2 , compared to placebo or 0.03 mg/kg E_2 , to decrease depression-like behavior (decrease immobility in the forced swim task). Both dosages of E_2 enhanced sexual behavior (i.e., lordosis) and increased motor behavior compared to placebo. The specificity of these effects was supported by little evidence of E_2 's dose-dependency for sexual or general motor behavior. Rats administered DMBA, compared to no carcinogen exposure, had increased tumor burden, an effect amplified by E_2 . E_2 dose-dependently increased uterine weight. 0.09 mg/kg E_2 produced physiological circulating levels of E_2 akin to levels in naturally receptive rats, compared to lower E_2 regimen (0.03 mg/kg) or placebo. Thus, E_2 dose-dependently decreased anxiety-like and depression-like behavior of female rodents; however, these same regimens can have unfavorable effects to increase tumorigenic capacity in reproductive tissues. Studies investigating E_2 's actions for these effects are ongoing.

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P63-14: STABLE EXPRESSION OF HUMAN VITAMIN D RECEPTOR IN MURINE VITAMIN D RECEPTOR KNOCK-OUT CELLS RESTORES 1,25D-MEDIATED GROWTH INHIBITION

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The vitamin D metabolite $1,25(\text{OH})_2\text{D}_3$ (1,25D) exerts pro-differentiating and anti-proliferative actions through the vitamin D receptor (VDR), which is expressed in normal and transformed mammary cells. Both vitamin D deficiency and VDR ablation alter mammary gland morphology and carcinogenesis in animal models, and vitamin D status inversely correlates with breast cancer risk in human populations. The goal of this project is to clarify the underlying mechanisms by which vitamin D and the VDR mediate anti-proliferative signaling in mammary cells, specifically, the relative contributions of genomic and non-genomic mechanisms of action.

Toward this goal, we have established a model system composed of transformed mammary epithelial cell lines derived from VDR knock-out (KO) mice and their wild-type (WT) littermates. WT145 cells, derived from normal mice, express transcriptionally active VDR and undergo growth arrest and apoptosis when treated with doses of 1,25D as low as 1 nM. In contrast, KO240 cells, derived from VDRKO mice, do not express VDR and are neither growth arrested nor rendered apoptotic in response to 1,25D, even at doses as high as 1 mM.

To examine the role of VDR signaling in 1,25D-mediated growth regulation, we stably transfected KO240 cells with a pSG5-hVDR-hygro vector to drive high-level expression of full-length human VDR. The resulting cell line, designated KO^{hVDR} cells, was

further subcultured to generate two subclones designated KO^{hVDR}F-B5 and -C5. KO240 cells stably expressing the empty vector (KO^{EV} cells) were also created as negative control. Western blotting indicated that the level of human VDR expressed in KO^{hVDR}F-B5 and -C5 cells is equivalent to the level of endogenous VDR expression in WT145 cells. Furthermore, induction of a VDR-driven reporter gene (CYP24) by 1,25D in -B5 and -C5 cells expressing human VDR is comparable to that in WT145 cells that endogenously express murine VDR. Growth assays indicate that 1,25D as well as synthetic VDR agonists effectively inhibited growth of KO^{hVDR}F-B5 and -C5 cells but had no effect on growth of the parental KO240 cells or the KO^{EV} negative control cells. These data indicate that human VDR is transcriptionally competent when stably expressed in mouse-derived mammary tumor cells. Furthermore, re-expression of heterologous human VDR restores the sensitivity of mouse tumor cells to the anti-proliferative effects of 1,25D.

To test the possibility that VDR mediates effects on cell behavior via ligand-independent mechanisms, KO240 cells stably expressing VDRs bearing mutations that abolish ligand binding have been created and tested in vitro. To explore the role of transcriptional regulation in vitamin D-mediated cancer cell growth and invasion, KO240 cells have been engineered with mutant VDRs that lack the ability to bind and activate consensus vitamin D response elements (VDRE). These novel cell lines bearing wild-type and mutant VDRs will be further exploited in xenograft models to assess whether in vivo effects are maintained in the tumor microenvironment.

In summary, our studies have confirmed that VDR is the target for 1,25D-mediated growth inhibitory effects in mammary tumors and will continue to provide mechanistic understanding that may be useful in designing novel vitamin D-based therapeutics for breast cancer.

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P63-15: RETINOL-RETINOIC ACID HOMEOSTASIS IS INTERTWINED WITH HUMAN MAMMARY EPITHELIAL CELL MORPHOGENESIS

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Retinoic acid (RA), that regulates cell proliferation, differentiation, and apoptosis acting via the retinoic acid receptors (RARs), is the bioactive derivative of dietary vitamin A (retinol). Animal studies have shown that RA-RAR signaling is required to maintain the homeostasis of mouse mammary gland morphogenesis. In a series of mechanistic studies we found that (1) disruption of RA-RAR signaling in untransformed human mammary cells leads to aberrant acinar morphogenesis in three-dimensional basement membrane; (2) disruption of RA-RAR signaling can be consequent to either the loss or unfunctional CRABP2, the protein necessary for transporting RA onto the RARs; and (3) disruption of RA-RAR-mediated epigenetic regulation of transcription leads to transcriptional downregulation of CRBP1, the protein required for both intracellular retinol transport and proper acinar morphogenesis of human mammary epithelial cells. Thus, lack of proper RA transport via CRABP2 onto the RARs, negatively affects RAR-regulated CRBP1 transcription. Apparently, retinol-RA homeostasis is intertwined via the RARs with human mammary epithelial morphogenesis.

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P63-16: ESTROGEN-MEDIATED REPRESSION OF REPRIMO—ROLE OF ESTROGEN RECEPTOR ALPHA AND HDAC7

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Estrogen-bound estrogen receptor α (ER α) can regulate the transcription of a large number of genes. Although the role of estrogen-mediated activation of genes is well established, the relevance of repression of genes is only beginning to be appreciated. We sought to identify key estrogen-repressed genes and the mechanism of their repression. We identified several primary ER α target genes that are repressed by estrogen using real-time quantitative PCR. One gene that particularly piqued our interest is Reprimo (RPRM) because of its role in p53-induced G2 cell cycle arrest. Additionally, it is highly methylated in a variety of cancers including breast cancer and maps to a locus that displays loss of heterozygosity, suggesting that it may be a tumor suppressor gene. As a result, E2-mediated repression of this gene may be a crucial step in the progression of breast cancer. RPRM is actively and vigorously repressed by estrogen and this repression is not due to a quicker turnover of the RPRM mRNA by estrogen. E2-mediated repression of RPRM levels does not require new protein synthesis since it is also repressed in the presence of the translation inhibitor, cycloheximide. Importantly, RPRM repression is ER α dependent as knockdown of the receptor abrogates the repression. Treatment with an HDAC inhibitor, Trichostatin A, completely abrogates the E2-mediated repression suggesting the involvement of HDAC's in this repression. More specifically, we show that HDAC7 is the critical HDAC involved in this repression. Remarkably, knockdown of HDAC7 affected the repression of about a third of the estrogen-repressed genes tested. Further examination of the interplay between HDAC7

and ER α revealed that they interact with each other. Moreover, HDAC7 expression levels are significantly higher in ER-positive tumors compared to ER-negative tumors across multiple datasets. Chromatin immunoprecipitation assays demonstrate that both ER α and HDAC7 are recruited to the RPRM promoter in the presence of estrogen. Importantly, knockdown of RPRM resulted in a doubling of the S phase suggesting that estrogen can regulate the cell cycle in part by repressing RPRM. Our study provides a comprehensive analysis of estrogen-mediated repression of genes and uncovers a striking requirement for HDAC7 thereby establishing a previously unknown link between HDAC7 and ER α . The HDAC7-mediated repression of ER α target genes may be critically involved in the progression of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0303.

P63-17: ERAP75 FUNCTIONS AS A COACTIVATOR TO ENHANCE ESTROGEN RECEPTOR α TRANSACTIVATION IN BREAST CANCER CELLS

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The estrogen receptor (ER) requires distinct coregulators for the efficient transcriptional regulation. We have recently identified ERAP75, Homo sapiens coiled-coil domain containing 62 (CDC62) protein, as a novel ER α coactivator. It has been reported that ER α expressed and functioned in the breast and plays a key role in the development of breast cancer. Here, we examined the role of ERAP75 as a coactivator for ER α in the breast cells. Using mammalian two-hybrid, GST pull-down, and coimmunoprecipitation methods, we show that ER α could interact with the C terminus of ERAP75 via its ligand-binding domain both in vivo and in vitro. The conserved LXXLL motif within the C terminus of ERAP75 is required for the interaction between ER α and ERAP75. ERAP75 could enhance ligand-dependent ER α transactivation in a dose-dependent fashion. Furthermore, using ectopic expression, we show that ERAP75 can enhance the expression of the endogenous ER α target gene, SDF-1, in breast cancer cells. Together, these results suggest that ERAP75 may function as a novel coactivator that can modulate ER α function. The understanding of the mechanism of ER α transactivation could possibly help to develop new strategies to control or treat breast cancer by targeting its transactivation protein complex.

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P63-18: GENOME-WIDE SCREENING REVEALS AN ESSENTIAL ROLE OF p27kip1 IN RESTRICTION OF BREAST CANCER PROGRESSION

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The genetic changes and mechanisms underneath the progression of estrogen-dependent to estrogen-independent, antiestrogen-resistant, and metastatic breast cancers are unclear despite the fact that they are a major problem of endocrine therapy. To identify genes responsible for this progression, we carried out a genetic screening by an enhanced retroviral mutagen (ERM)-mediated random mutagenesis in the estrogen-dependent T47D breast cancer cells. We found that T47D cells contain only one p27kip1 (p27) allele coding for the p27 cyclin-dependent kinase inhibitor. An ERM insertion into the p27 locus of T47D cells disrupted the p27 gene and created estrogen-independent and antiestrogen-resistant breast cancer cells that still maintained functional estrogen receptors. Disruption of p27 in T47D cells resulted in several changes and most of these changes could be rescued by p27 restoration. First, CDK2 activity was increased in the absence of estrogen or presence of estrogen antagonists tamoxifen or ICI; second, AIB1, a cancer-overexpressed transcriptional coactivator, was hyperphosphorylated, which made AIB1 a better coactivator for E2F1; and third, Gab2 and Akt activity were increased following E2F1 overactivation, leading to a significant enhancement of cell migration and invasion. Furthermore, the p27-deficient cells, but not T47D control cells, developed lung metastasis in an ovarian hormone-independent manner when they were intravenously injected into nude mice. In sum, loss of p27 activated AIB1, E2F1, Gab2, and Akt, increased cell migration and invasion, caused antiestrogen insensitivity, and promoted metastasis of breast cancer cells. These findings suggest that p27 plays an essential role in restriction of breast cancer progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0149 and National Cancer Institute (CA119689).

P63-19: ALTERED PROGESTERONE RECEPTOR ISOFORMS EXPRESSION IN MAMMARY CANCERS IN THE RAT

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Epidemiological studies have implicated progesterone (P) in increasing the breast cancer risk in women receiving combined estrogen (E) and P hormone therapy. In the

breast, P acts through two progesterone receptor (PR) isoforms. In the normal human adult breast, PRA and PRB are thought to be expressed in equimolar ratios, whereas in many breast cancers PRA expression is predominant and is associated with a poor prognosis for survival. In the rat mammary gland, PRA and PRB are often coexpressed in the same epithelial cells (20%–30% of luminal cells); however, PRB is a predominant isoform and up to 50% of luminal cells express only PRB. In contrast, PRA only expressing cells are rare. In the present study we tested whether mammary cancer development in the rat is associated with an alteration of PRA and PRB expression. Mammary cancers in Sprague-Dawley female rats were induced with DMBA (100 mg/kg body weight) given intragastrically at 50 days of age. Immunohistochemical analysis of PRA expression in tumors revealed ~ 2-fold increase in the percentage of PRA+ cells. In adjacent non-tumorous tissue, PRA expression was not increased and was comparable to the levels in the untreated control adult virgin gland. Conversely, the percentage of PRB+ cells in tumors decreased and the percentage of PRB only expressing cells was significantly lower than in normal glands. The proliferation rate in tumors was significantly higher than in adjacent normal tissue. Double immunofluorescence labeling showed that, in contrast to the normal mammary gland, a large percentage of proliferating cells were PRA+ and smaller proportion of proliferating cells expressed PRB. Labeling with PRA-, PRB-, and BrdU- specific antibodies revealed that of the proliferating PRA+ cells, 66% did not express PRB. Additionally, some PRB-PRA+ cells express telomerase reverse transcriptase (TERT), a main determinant of telomerase activity. Telomerase activity is a putative marker of normal stem cells and is expressed in cancer cells. We hypothesize that a subpopulation of PRA+ cells has mammary progenitor cell characteristics and is a target for mammary cancer initiation. Additionally, our results indicate that the subpopulation of PRA+PRB- cells is expanded in mammary cancers and actively proliferate. We speculate that PRA+PRB- cells may be putative progenitor cells in normal mammary gland that express TERT and upon carcinogen exposure become transformed leading to tumor development.

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P63-20: IDENTIFICATION OF HYPOXIA-INDUCIBLE FACTOR INHIBITORS

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Hypoxia contributes to the development and progression of breast cancer. The effects of tumor hypoxia are mediated by hypoxia-inducible factor 1 (Hif-1). Hif-1 is a dimeric protein consisting of an oxygen regulated Hif-1 α subunit and a constitutively expressed Hif-1 β subunit. Hif is involved in controlling key aspects of tumor biology such as angiogenesis and altered energy metabolism through transcriptional regulation of genes involved in these processes. Thus, agents that inhibit Hif-1 signaling have therapeutic potential for inhibiting breast cancer progression and development. We screened and identified small molecule inhibitors that disrupted Hif-1 transcriptional activity by different approaches. Some of these compounds inhibited hypoxia-induced gene expression in cancer cells. We are evaluating the effect of Hif-1 transcriptional inhibitors on Hif-1 biological function in breast cancer.

Nur77 (also known as NGFI-B/TR3) is an orphan nuclear receptor belonging to the steroid/thyroid/retinoid superfamily of transcription factors. It is an immediate early-response gene and is induced by growth factors and retinoids. Nur77 can promote growth or death of cancer cells. The opposing activities of Nur77 in cancer cells are regulated by its location in the cell. Nur77 promotes proliferation of the cancer cells by working in the nucleus of the cell. Through its actions on the mitochondria, Nur77 kills cancer cells via programmed cell death. We identified a novel cross-talk between Nur77 and Hif-1 signaling that can be exploited to develop novel Hif-1 inhibitors. Pharmacological inhibitors of Hif-1 are also likely to have tumor-specific effects as tissue partial pressures of oxygen values are lower in tumors than in normal tissue and Hif is strongly activated by hypoxia in the tumor microenvironment.

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P63-21: THE TUMOR SUPPRESSOR, WWOX1, ATTENUATES WBP-2- AND YAP1-MEDIATED SYNERGISTIC ENHANCEMENT OF ER AND PR TRANSCRIPTIONAL ACTIVITIES

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Background and Objective: Estrogen receptor- α (ER) and progesterone receptor (PR) are ligand-induced transcription factors. Coactivators enhance transcription of the target genes and play important roles in mammary gland development and tumorigenesis. We cloned WW-domain binding protein-2 (WBP-2) as a selectively coactivator of ER and PR transactivation functions. WBP-2 contains three polyproline (PY) motifs, of which the third PY motif (PY3) is essential for its coactivation functions. PY motifs specifically interact with the WW domains of proteins like yes associated protein (YAP1) and WW-domain-containing oxidoreductase (WWOX1). Recently, YAP1 a

characterized transcriptional coactivator, has been classified as an oncoprotein. WWOX1 has been identified as a tumor suppressor. The main objective of this study is to understand the molecular mechanism of action of WBP-2 and its signaling partner(s) in ER and PR signaling and breast tumorigenesis.

Methods: A dot blot based WW domain array was used to identify possible WBP-2 interacting proteins. Report gene assays as well as endogenous target gene quantifications were used to analyze the coactivation functions of WBP-2, YAP1 and WWOX1. Chromatin immunoprecipitation (ChIP) and reChIP assays were performed to study the mechanism of action of WBP-2, YAP1 and WWOX1.

Results: We show that YAP1 and WWOX1 interact with WBP-2, and that the PY3 motif of WBP-2 is critical for this interaction. We also show that YAP1 acts as a secondary coactivator of ER and PR. However, the coactivation function of YAP1 is revealed only in the presence of wild-type WBP-2 and not with the PY3 mutant WBP-2. Furthermore, YAP1 and WBP-2 are recruited to the ER responsive pS2 promoter and they synergistically enhance the transactivation functions of ER and PR. This synergistic increase is abrogated in the presence of WWOX1, but WWOX1 is not recruited

to the pS2 promoter. Recruitment of WBP-2 to the pS2 promoter is strictly dependent on the physiological expression levels of YAP1; conversely, YAP1 recruitment is dependent on WBP-2 expression levels. This suggests that the interaction between WBP-2 and YAP1 is critical of the coactivation functions of both the proteins.

Conclusions: Based on our data, we propose a mechanistic model that the coactivation functions of oncogenic YAP1 is dependent on WBP-2 and that WWOX1 acts as a tumor suppressor by interacting with WBP-2 and retaining it in the cytoplasm, resulting in the attenuation of WBP-2 and oncogenic YAP1 mediated ER and PR transcriptional activities. This work represents the first instance, where a tumor suppressor (WWOX1) has been shown to inhibit the transcription activation functions of steroid hormone receptor coactivators (WBP-2 and YAP1). As this complex regulatory mechanism is dependent on the PY-WW interaction module, it presents an intriguing possibility of targeting this module for therapeutic intervention in breast cancer by designing peptide inhibitors that specifically disrupt the interaction between PY motifs and WW domains.

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ANGIOGENESIS

Poster Session P64

P64-1 NOTCH REGULATES THE ANGIOGENIC RECEPTOR VEGFR-3 IN ENDOTHELIAL CELLS AND BREAST TUMOR VESSELS

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Studies in mice have demonstrated that Notch signaling is essential for the proper patterning of blood vessels in the embryo. In human breast tumors, an activator of Notch, Delta-like 4 (Dll4) was found to be upregulated in the developing blood vessels, suggesting that Notch may also have a role in pathological blood vessel development. However, the mechanism by which Notch regulates this process is poorly understood. In three different primary endothelial cell lines, we found that Notch signaling induced the expression of VEGFR-3, a cell surface receptor that responds to vascular endothelial growth factor C (VEGF-C) and functions in angiogenesis and lymphangiogenesis. VEGFR-3 signaling has also been implicated as a positive regulator of blood and lymphatic vessel development in both the embryo and breast cancer. Therefore in breast cancer, we hypothesize that Notch may regulate blood and/or lymphatic vessel development via its induction of VEGFR-3. The effectors downstream of Notch in the vasculature are poorly understood. Here, we report that Notch induces VEGFR-3 expression in primary blood endothelial cells and the embryonic vasculature. Notch, via its induction of VEGFR-3, made endothelial cells more responsive to VEGF-C promoting endothelial cell survival and morphological changes. In endothelial cells, Notch induced VEGFR-3 regulatory region reporters while mutating the Notch responsive elements disrupted transactivation by Notch. In complex with CSL, Notch binds to the VEGFR-3 promoter resulting in the acetylation of Histone 3. Thus, Notch functions to directly regulate VEGFR-3 expression in blood endothelial cells and promotes an angiogenic function for VEGFR-3. We further found that Notch1 and Notch4 were expressed in the lymphatic endothelium. Moreover, Notch1 is actively signaling in the extratumoral lymphatics of invasive mammary micropapillary carcinoma. Therefore, Notch signaling may also play a role in tumor lymphangiogenesis.

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P64-2: ESTROGEN-MEDIATED CELLULAR REMODELING USING BM-EPCS: NOVEL THERAPEUTIC TARGET FOR BREAST CANCER

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Neovascularization or angiogenesis is critical in sustaining growth of tumors as well as subsequent metastases. Circulating bone marrow-derived endothelial progenitor cells (BM-EPC) have been observed to contribute to neovascularization of tumors, and the identification of cellular and molecular mediators will impact clinical care. In this pilot program, we focused on developing an animal model that could examine the contribution of bone marrow-derived progenitor endothelial cells (BM-EPC) on neovascularization. Further, since estrogen is a major modulator of breast cancer initiation and progression and that estrogen affects BM-EPC migration, we examined the contribution of the BM-EPC induced neovascularization. Our animal model was designed to test the hypothesis

"Estrogen mobilizes circulating BM-EPC which home to the implanted breast carcinomas and promote tumor neo-vascularization." We utilized Tie2/GFP-Balb/c \pm ovariectomized \pm estrogen supplementation. These mice were transplanted with bone marrow derived from Tie2/GFP mice that were used as donors. Tumors were induced in these mice by surgical implantation of Tg1 or 4T1 (ATCC) murine mammary adenocarcinoma cells (derived from syngeneic BALB/c mice; 2×10^6 cells/0.3 ml PBS) into the fourth inguinal mammary gland after clearing the fat pad region of BMT mice. At the end of the experimental period, tumor incidence and progression were monitored by immunohistochemical analysis, and the BM-EPCs mobilization at the tumor site was measured and correlated with capillary density. We observed the concomitant mobilization of GFP and CD133 (marker of EPC) double-positive cells at the tumor site. Comparison of estrogen-supplemented and nonsupplemented groups revealed that E2 supplementation enhances both mobilization of GFP-CD133+ cells (EPCs) in the tumors as well as mobilized EPCs physically integrate into neovasculature resulting in significantly higher capillary density. The contribution of estrogen in angiogenesis and tissue remodeling, which are two processes indispensable for the growth of tumors, was also observed through Q-RT-PCR experiments on excised tumor-inoculated mammary tissues, in which the transcripts of various angiogenic cytokines involved in these two critical processes were significantly increased. E2-stimulated EPCs were also observed to secrete paracrine factors, which increased the proliferation and migration of 4T1 tumor cells. Last, EPCs isolated from BM were observed to form tube-like structures (tubulogenesis) upon addition of media obtained from E2-stimulated tumor cells. In conclusion, we have discovered a novel role of estrogen-stimulated BM-EPCs in tissue remodeling in a breast cancer animal model and presumably a novel therapeutic target.

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P64-3: THE ROLE OF CAPILLARY MORPHOGENESIS GENE 2 IN BREAST CANCER NEOVASCULARIZATION

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In this study, we demonstrate that Capillary Morphogenesis Gene 2 (CMG2), a putative angiogenic regulator, is expressed on breast tumor vessels. CMG2 was first identified as a gene with up-regulated expression during capillary morphogenesis of endothelial cells in vitro. The CMG2 gene encodes a type I transmembrane cellular receptor for anthrax toxin; however, endogenous CMG2 receptor function has yet to be defined. Several lines of evidence suggest that CMG2 may be an angiogenic regulator including: (1) CMG2 is expressed in primary endothelial cells, and (2) type IV collagen and laminin, the components of vessel basement membranes, may be endogenous ligands for CMG2. It is unclear how CMG2 might function during tumor angiogenesis. Thus, the objective of this training grant is to determine the role of CMG2 in the vasculature of tumorigenic breast tissue. It is hypothesized that CMG2 is able to positively regulate various angiogenic responses in tumor associated endothelial cells via interaction with extracellular matrix proteins. Consequently, the mechanism of action of CMG2 may be essential to breast tumor neovascularization, growth, and metastasis in vivo. (1) CMG2 is expressed on vessels of normal and tumorigenic breast tissue. In order to fully characterize CMG2 expression in breast cancer, immunohistochemistry was used to analyze normal human mammary epithelium and various subtypes of human breast tissues. Immunohistochemical analysis of invasive ductal carcinomas revealed that CMG2 expression was restricted to vessels and tumor stroma. Analysis of normal breast tissue revealed that CMG2 expression was restricted to the basement membranes surrounding mammary ducts and to vessels within the tissue. Furthermore, CMG2 was found to colocalize with type IV collagen and laminin in all of the samples analyzed. Taken together this data suggests that CMG2 is an important factor during physiological and pathological angiogenesis. (2) CMG2 expression is required for angiogenesis in vitro. The investigation of CMG2 as an angiogenic regulator was achieved by engineering primary human umbilical venous endothelial cells (HUVEC) to ectopically express vector-based small interfering RNAs (siRNA) that target CMG2 knockdown. These cells were then used with in vitro assays designed to model the different steps of angiogenesis. CMG2 siRNA specifically reduced CMG2 expression at mRNA and protein levels in HUVEC by up to 50%. This reduction in CMG2 expression resulted in the significant inhibition of HUVEC proliferation as compared to control cells expressing scrambled siRNA. CMG2 knockdown also disrupted HUVEC capillary-like network formation and tube formation as compared to control. This data suggests that an appropriate level of CMG2 signaling is necessary to make fine networks of neovessels. In conclusion, these studies provide the first demonstration of CMG2 expression on the vasculature of normal and tumorigenic human breast tissue suggesting that CMG2 is likely to be an important factor during breast cancer neovascularization and provide evidence that CMG2 may function to promote endothelial cell proliferation and tube morphogenesis, two important angiogenic processes. The data generated thus far points to CMG2 as an attractive target for new therapeutics designed to inhibit angiogenesis during breast tumor formation. Future work will determine whether a CMG2 receptor decoy could function as one such therapeutic.

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P64-4: A NOVEL GLYCOTHERAPEUTIC FOR CURING BREAST CANCER

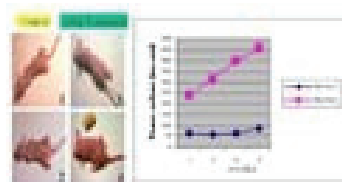
Dipak K. Banerjee,¹ Aditi Banerjee,¹ Juan A. Martinez,¹ Mien-Chie Hung,² and Krishna Baksi¹

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Breast cancer affects women globally. In the United States 211,240 new breast cancer cases were diagnosed in the year 2006 and 40,410 have died from the disease. Complex etiology of the disease needs personalized treatment for elimination. Angiogenesis is critical for the breast tumor growth and requires active participation of asparagine-linked glycoproteins. We have evaluated the requirement of lipid-linked oligosaccharide (LLO, Glc₃Man₉GlcNAc₂-PP-Dol) in angiogenesis and asked if inhibiting the LLO biosynthesis, i.e., a glycotherapeutics could eliminate the breast tumor growth. Our model is a nontransformed capillary endothelial cell line with preserved physiology and anatomy that respond adequately to cellular microenvironment. Enhancing both LLO biosynthesis and protein N-glycosylation with cAMP-related signaling resulted in accelerated cell cycle progression, increased capillary lumen formation, and HSP-70 and HSP-90 expression. Mannosylphospho dolichol synthase (DPMS), a key regulator in the LLO biosynthesis also increased. Mechanism supported PKA-dependent phosphorylation. Cloning and sequencing identified a PKA motif in DPMS gene. TM (a glucosamine-containing pyrimidine nucleoside and an LLO inhibitor) inhibited the cell surface N-glycan expression, arrested the cells in G1 causing a down regulation of Bcl-2, D-type cyclins, cdk, and transcription factor E2F expression and consequently halting angiogenesis. High level of caspase-3, -9, and -12 expression as well as increased DNA laddering and Annexin V binding all supported apoptosis. High expression of GRP-78/Bip indicated "ER stress" and the induction of unfolded protein response.

There was no detectable DPMS activity and/or DPMS protein expression, and cells lost the protection of VEGF₁₆₅. Excitingly, TM reduced the breast tumor xenografts by 50%–60% in 3–4 weeks when administered intravenously or orally in nude mice developed either with MDA-MB-435 or with MDA-MB-231 human breast cancer cells (Figure). Therefore, antibiotic TM could succeed in the clinic as an excellent breast cancer therapeutic.

Banerjee, D.K., Martinez, J.A. and Bakshi, K. (2007) Significance of protein N-glycosylation in breast tumor angiogenesis. In: *Angiogenesis: Basic Science and Clinical Applications* (Eds. M.E. Maragoudakis and E. Papadimitriou), pp 1-23, Transworld Research Network, Trivandrum, Kerala, India.



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P64-5: TARGETED MOLECULAR THERAPY OF BREAST CANCER-DERIVED BRAIN METASTASES

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Breast cancer is the most common malignant disease in Western women and one of the most frequent cancers to develop secondary brain tumors. In these patients, it is not the primary tumor but the metastases at distant organs that are the main cause of death. Nearly 1.3 million people diagnosed with cancer in the United States each year will develop brain metastases. This complication is usually viewed as a consequence of progressive metastatic disease, for which few effective treatment options exist. Because of the blood-brain barrier and the unique microenvironment of the brain, novel and distinct therapeutic approaches for brain metastases prevention and treatment need to be developed. Receptors present in cell surfaces can be exploited as ligand-directed systems that enable targeted delivery of drugs and imaging agents to tumors. Over the past decade, the use of combinatorial approaches toward a new ligand-directed pharmacology to deliver vascular-targeted cytotoxics in tumor models has evolved significantly and has even been adapted for direct application in cancer patients. We hypothesized that a restricted set of receptors is specifically expressed on blood vessels of breast cancer-derived brain metastases. We proposed to exploit the molecular diversity of cell surface receptors expressed on blood vessels of brain metastases that will eventually result in a ligand-receptor functional map for targeted delivery. We used the random phage-display library CX7C (all combinations of seven amino acids flanked at each end by cysteine residues) to isolate targeted peptides to angiogenic vasculature of brain tumors in vivo and successfully isolated tumor-vasculature targeted peptides. As proof-of-principle, we coupled the leading peptide to a hybrid vector composed of genomic elements from adeno-associated virus (AAV) and an M13-derived phage. AAV/phage (AAVP) chimeras display tumor-homing peptides that are suited for Herpes simplex virus thymidine kinase (HSVtk) and TNF-mediated treatment at the molecular level. We were able to suppress tumor growth in a preclinical model. These approaches are of potential major significance for several reasons. In the current era of emerging molecular therapy, there is a compelling need to develop improved means to treat and image brain tumors. In addition, the development of novel methodologies for imaging may present additional targets for selective and less toxic targeted molecular therapeutics. Finally, such newer molecular therapies that are being developed for treatment of brain tumors may prove to be useful in other diseases where comparable molecular derangements may also be identified as being relevant for both molecular imaging as well as for molecular targeted therapy.

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P64-6: OVEREXPRESSION OF VEGF BY MCF-7 BREAST CANCER CELLS PROMOTES ESTROGEN-INDEPENDENT TUMOR GROWTH IN VIVO

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Alteration of the phenotype of breast cancers from estrogen-dependent to estrogen-independent growth often leads to the failure of anti-estrogenic tumor therapies. We report that overexpression of VEGF by estrogen-dependent MCF-7 breast cancer cells could abolish estrogen-dependent tumor growth in ovariectomized mice. In the absence of estrogen, MCF-7 VEGF-expressing tumors with increased vessel density showed growth kinetics similar to, or even greater than, that of parental MCF-7 tumors with estrogen-supplementation. Overexpression of VEGF by MCF-7 cells or treatment on parental MCF-7 cells with recombinant VEGF also stimulated cell proliferation in cul-

ture. Our data suggest that VEGF stimulation of MCF-7 tumor angiogenesis and growth is mediated by both autocrine and paracrine mechanisms.

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P64-7: INTRACRINE SURVIVAL OF HUMAN BREAST CARCINOMA CELLS BY VEGF, VEGFR-1 AXIS

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Vascular endothelial growth factor (VEGF) expression in breast tumors has been correlated with a poor outcome in the pathogenesis of breast cancer. However, the expression, localization, and function of VEGF receptors VEGFR1 (also known as FLT1) and VEGFR2 (also known as KDR or FLK1), as well as neuropilin 1 (NRP1), in breast cancer are controversial. We investigated the expression and function of VEGF and VEGF receptors in breast cancer cells. We observed that while VEGFR1 expression was abundant, VEGFR2 expression was low, and NRP1 expression was variable. MDA-MB-231 and MCF-7 breast cancer cells, transfected with antisense VEGF cDNA or with siVEGF (VEGF-targeted small interfering RNA), showed a significant reduction in VEGF expression and increased apoptosis as compared to the control cells. Additionally, specifically targeted knockdown of VEGFR1 expression by siRNA (siVEGFR1) significantly decreased the survival of breast cancer cells through down-regulation of protein kinase B (AKT) phosphorylation while targeted knockdown of VEGFR2 or NRP1 expression had no effect on the survival of these cancer cells. VEGFR1 was predominantly expressed internally in MDA-MB-231 and MCF-7 breast cancer cells. Specifically, VEGFR1 was found to be colocalized with lamin A/C and was expressed mainly in the nuclear envelope in breast cancer cell lines and primary breast cancer tumors. This study provides evidence of a unique survival system in breast cancer cells by which VEGF can act as an internal autocrine (intracrine) survival factor through its binding to VEGFR1. These results may lead to an improved strategy for tumor therapy based on the inhibition of angiogenesis and inhibition of tumor growth.

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P64-8: THE UNEXPECTED STRIDES OF ANGIOGENESIS

Lyuba Varticovski
National Cancer Institute

The award from the Department of Defense many years ago enabled me to pursue an area of research that, at the time, was at least heretical. The IDEA award entailed understanding mechanisms of action and signal transduction pathways of angiopoietins, newly discovered ligands for Tie receptor. More than a dozen publications stemmed from that work. My laboratory continues to work on development of novel cancer therapies, and use of genetically engineered mouse models for translational research. We continue to revisit the role of angiogenesis in tumor progression and response to therapies. Our recent work involves the mechanisms of drug resistance specifically in a population of tumor-initiating cells that have cancer stem cell properties. These cancer stem cells promote angiogenesis leading to accelerated tumor growth in vivo, whereas cells depleted of these cells do not elicit this response. In addition, BRCA1 deficient breast cancer contains more than one type of cancer stem cells, and these cells have similar characteristics, including stem cell gene expression and drug resistance. I am grateful to DOD for the IDEA award that allowed me to have a broader look at tumor biology that is useful in development of novel anticancer therapies.

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P64-9: T-CADHERIN IS REQUIRED FOR ADIPONECTIN ASSOCIATION AND VASCULAR SUPPORT OF MMTV-PYV-MT MAMMARY TUMORS

Barbara Ranscht,¹ Lionel W. Hebbard,¹ Martin Denzel,¹ Michele Garlatti,¹ Lawrence J. T. Young,² Robert D. Cardiff,² and Robert G. Oshima¹

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T-cadherin delineates endothelial, myoepithelial, and ductal epithelial cells in the normal mouse mammary gland and becomes progressively restricted to the vasculature during mammary tumorigenesis. To test the function of T-cadherin in breast cancer, we inactivated the T-cadherin gene in mice and evaluated tumor development and pathology after crossing the mutation into the MMTV-polyoma virus middle T (PyV-mT) transgenic model. We report that T-cadherin deficiency limits mammary tumor vascularization and reduces tumor growth. Tumor transplantation experiments confirm T-cadherin's stromal role in tumorigenesis. In comparison with wild-type MMTV-PyV-mT controls, T-cadherin-deficient tumors are pathologically advanced and metastasize to the lungs. T-cadherin is a suggested binding partner for high molecular weight forms of the circulating fat-secreted hormone adiponectin. We discern adiponectin in asso-

ciation with the T-cadherin-positive vasculature in the normal and malignant mammary gland and report that this interaction is lost in the T-cadherin null condition. Current work establishes the role of adiponectin in the MMTV-PyV-mT model. Our work raises the possibility that vascular T-cadherin - adiponectin association may contribute to the molecular crosstalk between tumor cells and the stromal compartment in breast cancer and present a novel therapeutic target.

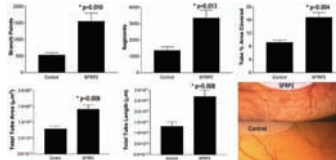
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0574.

P64-10: FUNCTIONAL CHARACTERIZATION OF THE NOVEL BREAST TUMOR ENDOTHELIAL MARKER, SFRP2, ON ANGIOGENESIS IN THE CHICK CHORIOALLANTOIC MEMBRANE IN VIVO AND IN VITRO

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Background: We sought to characterize molecular changes between breast tumor and normal vessels to identify genes that may serve as therapeutic targets. We performed immunohistochemistry (IHC) and laser capture microdissection of vessels from frozen human breast tumors and normal breast tissue, extracted and amplified RNA, and hybridized to 44K Agilent microarrays. Fifty-five genes had > 4 fold increased expression in tumor vessels, one of which was secreted frizzled-related protein 2 (SFRP2). We confirmed localization of SFRP2 to endothelium and increased expression in tumor vessels versus normal, using IHC with antibodies to SFRP2 on paraffin-embedded breast tumors. The purpose of this study is to characterize the contributions of SFRP2 to angiogenesis.

Methods: *Chick chorioallantoic membrane (CAM) Assay:* The chick embryo of day-4 fertilized eggs were placed into culture dishes and incubated. On day 8, filter disks with and without SFRP2 (100 ng) were placed on the CAM. The effect of SFRP2 on angiogenesis was examined on day 11. Pictures were taken with a Wild M-4 70 MacroSystem, and angiogenesis was quantified using Metamorph Software with an angiogenesis module. *Tube Formation:* Wells of a 96-well plate were coated with 100 μ L of Matrigel, which polymerized at 37°C for 1 hour. 1×10^4 mouse endothelial (MEC) cells / well in 150 μ L of DMEM with 10% FBS with and without SFRP2 (3-3000 pM) were seeded onto the matrix for 8 hours. Results were quantified by counting the number of branch points. *Scratch Wound Assay:* MEC cells were plated at 10,000 cells/well. The wound was formed using a 1 ml pipette tip and SFRP2 0.3 pM-300 pM or buffer alone was added to the wells. Migration was measured from 8 to 24 hours. *Statistics:* Statistical differences between SFRP2 and control were evaluated with an unpaired two-tailed Student's *t*-test.



Chick Chorioallantoic Membrane Assay

Results: *CAM Assay:* SFRP2 induced angiogenesis on the CAM (Figure). *Tube Formation Assay:* At 8 hours, the median number of branch points for control was 27 ± 17, and for SFRP2-treated cultures was 84 ± 5, $p < 0.001$. *Scratch Wound Assay:* At 16 hours, control cells migrated 480 μ m ± 23, and SFRP2-treated cells migrated 680 μ m ± 40, $p = 0.001$.

Conclusions: The novel breast tumor endothelial marker, SFRP2, stimulates angiogenesis on the CAM, and increases endothelial cell migration and tube formation in vitro. Based on its expression and function, SFRP2 may serve as a therapeutic target for developing inhibitors of the tumor vasculature.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0434 and National Institutes of Health.

P64-11: THICK-MATRIX CAPILLARY-PERFUSED BREAST TISSUE BIOREACTOR FOR STUDYING ANGIOGENESIS AND METASTASIS IN BREAST CANCER

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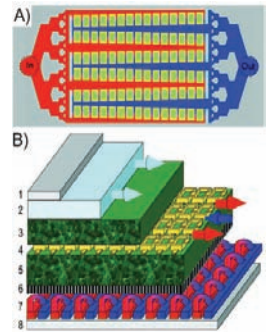
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The limitations of animal studies and in vitro cell culture techniques suggest the need for more realistic in vitro environments to identify mechanisms by which breast cancer tumors grow and spread and to assess antiangiogenic and antimetastatic drug efficacy.

We are developing a three-dimensional, capillary-perfused breast tissue bioreactor (PBTB) that will use open scaffolds to guide endothelial cells to form an arteriole-cap-

illary-venule geometry that will be connected to an external perfusion system. The PBTB will include ultraporous nanofilters with independent perfusion systems to maintain viability of a 3-D cell culture until the capillary system can deliver nutrients and remove metabolic byproducts from the interior of a thick cell culture containing multiple cell types.

As an initial step toward our goal, we have successfully established a 3-D organotypic culture system in which human microvascular endothelial cells form capillary tubes in a realistic connective tissue microenvironment containing collagen matrix and primary fibroblasts in a standard transwell. To direct the geometric pattern of vessel formation, we are currently testing a variety of scaffolding materials of varying rigidity including poly(dimethylsiloxane) (PDMS), photocurable epoxy (SU8), and collagen gel itself with direct embossing/patterning of vessel channels. In addition, we have built an initial bioreactor prototype that is currently being assessed for delivery of sufficient nutrient supply and metabolite exchange for 3-D tissue culture in a contained microfluidic environment. Furthermore, physical separation of the media delivery from the 3-D culture environment is necessary to prevent unintended effects on the vascular morphogenesis; however, effective diffusional transport of nutrients and waste products is also required. Ultraporous alumina nanofilters (with porosity >50%), which have a higher pore-to-filter density than other traditional filter materials such as polycarbonate, have been developed in-house and are being validated for enhanced diffusional transport and biocompatibility.



(A) Arteriole-capillary-venules (B) superfusion, collagen, capillary, collagen, ultrafilter, perfusion

When we can reproducibly pattern the endothelial morphogenesis, we will focus on establishing controlled external microfluidic connections to internally perfuse the bioreactor system through the biological capillary-bed system. Once fully developed, the individual components will be combined into one PBTB system that will more realistically mimic organ physiology. When developed and validated, the PBTB will be used in the future as a host environment for breast cancer cells to study antiangiogenic or other potential antitumor therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0507 and Vanderbilt Institute for Integrative Biosystems Research and Education.

P64-12: ENDOTHELIAL ADHESION IN FIBROBLAST-DERIVED 3-D MATRIX

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Cell behavior is regulated by the mechanical properties of the extracellular microenvironment. These mechanical signals modulate cell adhesion to extracellular matrix (ECM) and to neighboring cells thereby influencing proliferation, migration, morphogenesis, and invasion. We are interested in the effects of matrix mechanics on angiogenesis and malignant mammary epithelial metastasis in breast cancer. Fetal human lung fibroblasts were cultured for 7 days to generate a cell-derived three-dimensional matrix, which was voided of cells by detergent lysis. This matrix was primarily comprised of fibronectin and was approximately 5 μ m thick. Matrix mechanics and endothelial cell adhesion within these matrices were investigated. Utilizing atomic force microscopy (AFM), the elastic modulus of the matrix was determined to be approximately 1.5 kPa. Human umbilical vein endothelial cells (HUVEC) were seeded onto the matrix and studied using immunofluorescence imaging, deconvolution, and 3-D reconstruction. The three-dimensional cell-ECM adhesions formed by HUVEC on these matrices contained tyrosine-phosphorylated signaling proteins including Y397-FAK in addition to other matrix adhesion molecules such as vinculin. The volumetric ratio of colocalized to total adhesion molecules was used to quantify the percentage of a specific 3-D adhesion constituent directly associated with fibronectin. HUVEC seeded at higher density (2.1×10^5 cells/cm²) formed cell-cell adhesions containing both VE-cadherin and PECAM-1. After 12 hrs of culture, the cells began forming ring-like structures. The 3-D ECM produced by human lung fibroblasts provides a unique and effective bioscaffold for the study interactions between cells and matrix during angiogenesis. We plan to extend these investigations to the study of the impact of matrix mechanics on the growth behavior of malignant mammary epithelial cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0279 and National Institute of Dental and Craniofacial Research.

SIGNAL TRANSDUCTION II

Poster Session P65

P65-1: FLY TO MOUSE: A NEW APPROACH TO CANCER METASTASIS

Ross Cagan,¹ Marcos Vidal,² Emanuela Heller,² Lorena Salvaggione,² Lourdes Ylagan,² Mark Watson,² Mark Wilkins,² Jill Fink,² and Katherine Weilbaecher²
¹Mount Sinai School of Medicine, New York and ²Washington University

Cancer has proven a difficult disease to achieve significant long-term advances in patient survival; improvements in survival are often measured in months. Given this complexity, we have combined our approaches to take a broader, whole-animal approach to the complexities inherent in cancer and associated metastasis.

The Cagan laboratory has undertaken a genetic and drug screening approach targeting cancer and metastasis utilizing the fruitfly *Drosophila*. They have modeled several cancers including breast and thyroid and have identified several pathways that mediate overgrowth and metastasis. Further, careful analysis of discrete tumors has suggested a novel model in which metastasis is the outcome of early, local interactions between normal and transformed epithelial cells specifically at tumor boundaries. Using robotics, the Cagan laboratory has developed a method of high-throughput drug screening using robotics to screen *Drosophila* models of cancer and metastasis. This whole-animal approach helped identify a compound currently in Phase 3 clinical trials for medullary thyroid carcinoma and several candidate compounds that suppress metastasis in the fly.

Dr. Weilbaecher's laboratory has established in vivo models to explore the interactions between metastatic breast tumor cells and bone. As part of this effort, they have joined with the Cagan laboratory to identify new therapeutic targets and novel therapeutic compounds. Their evidence indicates that a compound identified as antimetastatic in *Drosophila* is an efficient inhibitor of lung metastasis in a murine xenograft model of breast cancer. Interestingly, the effects of this chemical inhibitor are more complex at the site of cell metastasis to the bone, indicating that these interactions alter the nature of the invading cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0360 and National Institutes of Health.

P65-2: A NOVEL SCREEN FOR SMALL MOLECULE MODULATORS OF THE Wnt SIGNALING PATHWAY

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 New York University School of Medicine

Rationale: The Wnt/wingless (wg) pathway is one of a core set of evolutionarily conserved signaling pathways that regulate many aspects of metazoan development. Inappropriate activation of the Wnt pathway has been associated with tumorigenesis of the liver, colon, breast, and skin. One of the most important effectors of the Wnt pathway is encoded by the transcription factor, beta-catenin (ss-cat)/armadillo (arm). Since catenin responsive transcription (CRT) has been implicated in the genesis of many cancers, it makes a good target for developing therapeutics that could modulate the nuclear activity of ss-cat. Recently, we employed a novel methodology of integrating a "sensitized" chemical genetic high-throughput screen (HTS) with RNA-interference (RNAi) technology in order to identify specific small molecule inhibitors of CRT in *Drosophila* and human cell lines. Our screening approach allows for the specific targeting of the activity of the signaling pool of ss-cat. In a screen of 15,000 compounds, we identified 34 candidate inhibitors of CRT using a luciferase reporter-based cellular assay. Our preliminary studies using a variety of reporters for other cell signaling pathways strongly suggest that some of the candidate compounds can specifically modulate the activity of CRT. They are not cytotoxic at the doses that have been used for treatment of the cells, and their IC₅₀ are significantly low (nM-pM range). Additionally, these compounds can inhibit CRT in a wide range of cell types, including *Drosophila*, mouse, and human cells as well as a variety of Wnt/ss-cat-related/responsive cancer cell lines, such as the MCF7 human breast carcinoma cell line.

Study Design: To further assess the effect of the candidate small molecules on the activity of the Wnt signaling pathway, we propose to: (1) investigate the molecular

mechanism(s) by which the candidate small molecules (identified in the pilot screen) impact CRT and Wnt-responsive phenotypes in cultured cells; (2) expand the scope of the novel screening approach by conducting additional compound screens in specific Wnt-responsive mammalian and cancer cell lines using image-based high-content screening (HCS) for cell morphology, cell invasion, and proliferation-based phenotypes; (3) identify the comprehensive protein "interactome" network of ss-cat using the TAP-tag method and mass spectrometry; and (4) test whether the candidate compounds identified in the small molecule screen can influence binary interactions between ss-cat and its protein partners and use comparative phenotypic analysis of RNAi-mediated knockdown of ss-cat's protein interaction partners and those obtained from treatment of Wnt-responsive cells with candidate small molecules as a means for target identification.

Broader Significance: Our multipronged approach to target the activity of ss-cat (and similar screens in the future) will lead to the identification of specific drug inhibitors while also addressing their mechanism of action. These may serve as prototypes for the development of antitumor drugs targeted to ss-cat-responsive transcriptional programs involved in breast cancer as well as other CRT-related cancers. Importantly, the scope of the novel "integrated" screening technology to identify compound inhibitors would be applicable to various other cell-signaling pathways that have been implicated in the genesis of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0541.

P65-3: THE RECRUITMENT OF c-Src TO ErbB-2 CATALYTIC DOMAIN IS A CRITICAL EVENT IN ErbB-2 MEDIATED TRANSFORMATION

William Muller, Richard Marcotte, Calvin Roskelley, Lixin Zhou, and Harold Kim
 McGill University

Previous studies have demonstrated that c-Src tyrosine kinase interacts specifically with ErbB2 but not to other members of the EGFR family. To identify the site of interaction, we recently employed a chimeric EGFR/ErbB-2 receptor approach to show that c-Src binds specifically to the kinase region of ErbB2. Here we demonstrate that retention of conserved amino acid motif surrounding tyrosine 882 (referred to as EGFR^{YHAD}) is sufficient to confer binding to c-Src. We further show that the chimeric EGFR receptors that contain the Y882 motif is transforming in vitro and in vivo following ligand stimulation that was further correlated with sustained tyrosine phosphorylation of STAT3. Finally, we demonstrate that mutations in EGFR catalytic domain conferring resistance to gefitinib have generated EGFR variant catalytic domains that have regained their capacity to bind c-Src. These observations have important implications in understanding the molecular basis for resistance to EGFR inhibitors and implicate c-Src as critical in EGFR family-induced transformation.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0244 and Canadian Breast Cancer Research Alliance.

P65-4: INTEGRIN $\alpha 5 \beta 1$ CAUSES STABILIZATION OF CORTICAL ACTIN FIBERS VIA FOCAL ADHESION SIGNALING AND DOWNREGULATION OF RhoA GTP IN DORMANT BREAST CANCER CELLS IN AN IN VITRO MODEL

Robert Wieder and Judith Barrios
 University of Medicine and Dentistry of New Jersey Medical School

Breast cancer cells can metastasize very early in the disease to various sites, including the bone marrow. Interactions with the bone marrow stroma can potentially activate signaling that allows breast cancer cells to survive in a growth-arrested dormant state for prolonged periods. Accordingly, we have recently developed an in vitro model for cancer cell dormancy in the bone marrow in which integrin $\alpha 5 \beta 1$ promotes survival of dormant breast cancer cells. In the model, FGF-2, a growth factor synthesized and deposited in the bone marrow stroma, acts as a differentiation factor in estrogen-responsive breast cancer cells. Among the differentiating effects, FGF-2 induces growth arrest and re-expression of integrins, among which integrin $\alpha 5 \beta 1$ acts as a specific survival factor when bound to fibronectin in the bone marrow microenvironment. In the current study, we investigate a potential role of integrin $\alpha 5 \beta 1$ in promoting de novo cytoskeletal rearrangements and consequent cell spreading associated with dormant phenotype. Our results demonstrate that integrin $\alpha 5 \beta 1$ signaling causes focal adhesion kinase activation and focal adhesion complex stabilization, potentially leading to characteristic cytoskeletal morphology found in dormant cells. Specifically, we report that ligation and activation of integrin $\alpha 5 \beta 1$ promotes deactivation of the small GTPase Rho A, stabilization of actin and its redistribution to the cortex. Our study provides additional evidence that GRAF, a GTPase regulator associated with focal adhesion kinase (FAK), is present in the focal adhesions in these dormant cells. Our results implicate GRAF as a key factor modulating the dynamics of GTPase loading of Rho A in dormant cells, potentially contributing to their phenotype. Taken together, our data strongly suggests

sustained activation of a novel integrin $\alpha 5 \beta 1$ signaling pathway downstream to FGF-2 signaling contributing to the dormant phenotype of breast cancer cells in the bone marrow microenvironment.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0343.

P65-5: CELLULAR LOCALIZATION OF THE ACTIVATED EGFR DETERMINES ITS EFFECT ON CELL GROWTH

Brian P. Ceresa and Dustin Hyatt

University of Oklahoma Health Sciences Center

The epidermal growth factor (EGF) receptor (EGFR) is a ubiquitously expressed receptor tyrosine kinase that regulates diverse cell functions that depend on cell type, the presence of downstream effectors, and receptor density. In addition to activating biochemical pathways, ligand stimulation causes the EGFR to enter the cell via clathrin-coated pits. Endocytic trafficking influences receptor signaling by controlling the duration of EGFR phosphorylation and coordinating the receptor's association with downstream effectors. To better understand the individual contributions of cell surface and cytosolic EGFRs on cell physiology, we used EGF that was conjugated to 900 nm polystyrene beads (EGF-beads). EGF-beads can stimulate the EGFR and retain the activated receptor at the plasma membrane. In MDA-MB-468 cells, a breast cancer cell line that overexpresses the EGFR, only internalized, activated EGFRs stimulate caspase-3 and induce cell death. Conversely, signaling cascades triggered from activated EGFR retained at the cell surface inhibit caspase-3 and promote cell proliferation. Thus, through endocytosis, the activated EGFR can reciprocally regulate cell growth in MDA-MB-468 cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9367 and American Cancer Society.

P65-6: THE ACTIVATION KINETICS OF ERK1,2 DETERMINES THE MORPHOLOGICAL RESPONSE IN MAMMARY DUCTS

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¹City University of New York, College of Staten Island, ²Lawrence Berkeley National Laboratory, and ³University of California, San Francisco

A fundamental question of signal transduction is how the canonical components of the MAPK pathway, Raf \rightarrow MEK \rightarrow ERK, integrate signals from multiple stimuli into distinct cellular outcomes. To address this question, we examined the morphological outcome of mammary ducts stimulated *ex vivo* with either transforming growth factor- α (TGF α) and/or fibroblast growth factor-7 (FGF7). We show that a sustained activation of ERK1,2 for 1 hour, induced by TGF α , was necessary and sufficient to initiate branching morphogenesis, whereas a transient activation for 15 minutes, induced by FGF7, led to growth without branching. Unlike TGF α , FGF7 promotes sustained proliferation as well as ectopic localization of, and increase in, keratin-6-expressing cells. Simultaneous stimulation by FGF7 and TGF α resulted in growth without branching, suggesting that the FGF7-induced ERK1,2 signaling and associated phenotypes are dominant. FGF7 may prevent branching by suppression of two necessary TGF α -induced morphogenetic effectors, matrix metalloproteinase-3 (MMP-3/stromelysin-1) and fibronectin. These findings show that expression of morphogenetic effectors, proliferation, and cell-type decisions during tissue morphogenesis are dependent on growth factor-specific ERK1,2 activation kinetics. To further address the relevance of ERK1,2 activation kinetics during morphogenesis, we show how mammary ducts respond to inappropriate activation of ERK1,2 by use of an inducible active RAF. These latter findings provide further evidence that both cellular and tissue response is intimately tied with the duration of MAPK activation and that a small percentage of cells with a constitutively active MAPK pathway can deregulate the entire morphogenetic tissue response.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0486.

P65-7: UNDERSTANDING THE ROLE OF RhoC GTPase IN INFLAMMATORY BREAST CANCER: A DUAL MATHEMATICAL-EXPERIMENTAL STUDY

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The most damaging change during cancer progression is the growth of metastases. Understanding genes that drive metastasis and how to correctly inhibit them is paramount to improve survival. The protein RhoC GTPase was found to be crucial in that process in different cancers, particularly in a highly aggressive form of breast cancer termed inflammatory breast cancer (IBC). RhoC is a molecular switch cycling between inactive (GDP-bound) and active (GTP-bound) states, tightly regulated by several regulatory proteins.

We have developed a dual mathematical-experimental approach to understand the mechanism of RhoC deregulation in breast cancer. A major impact of this work is, for the first time, to quantitatively predict the effects of drugs targeted against RhoC in cancer. The methodologies include biochemical experiments for active and total RhoC, quantitative imaging, and mathematical modeling.

We have found that the activation profile of RhoC in IBC cells (SUM149) after stimulation with lysophosphatidic acid (LPA) is similar to those of other small GTPases. Unexpectedly, however, a significant and rapid increase in RhoC protein abundance accompanies this activation profile. Several tests and alternative experiments were performed to ensure that this last observation was not an experimental artifact.

The bacterial exotransferase from *Clostridium botulinum* (C3 transferase) irreversibly ADP-ribosylates RhoA, RhoB, and RhoC proteins and inhibits their downstream signaling interactions. After a recovery period following C3 transfection, SUM149 cells were stimulated with LPA. RhoC activation was effectively suppressed by C3 as expected. Interestingly, the increase in total protein level was also blocked by C3. These data suggest that inhibition of RhoC activation by C3 concurrently suppressed the increase in RhoC protein. The pathway initiated by LPA that produces the increase in total RhoC is therefore dependent on RhoC-GTP, revealing the existence of a previously unrecognized positive feedback loop between the activation cycle and the protein synthesis-degradation balance for RhoC.

RhoC protein half-life was measured after treatment with the protein synthesis inhibitor cycloheximide (CHX) in a time course experiment with SUM149 cells. The half-life of the protein was estimated around 40 minutes. Less protein degradation was observed in cells pretreated with LPA than in cells treated with CHX only. In addition, RhoC protein production rate was measured after treatment with the proteasome inhibitor MG132. Dual LPA and MG132 treatment indicates that this production rate is not modified by LPA. The CHX and MG132 experiments indicate that the increase in protein level after LPA stimulation is due to a decrease in the protein degradation rate, and the C3 experiment indicates that this mechanism is mediated by RhoC-GTP.

We have developed a set of ordinary differential equations to describe RhoC-GTP and RhoC protein temporal courses. The equations take into account the mentioned positive feedback loop between the activation cycle and the control of the protein degradation rate. The contribution of this regulatory mechanism to the aggressive IBC phenotype is studied, as well as its impact in planning RhoC-directed drug strategies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0489; Burroughs Wellcome Funds; the Breast Cancer Research Foundation; and National Institutes of Health (R01 CA77612).

P65-8: THE ROLE OF RAL G-PROTEINS IN AUTOPHAGY

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The Ras-like guanyl nucleotide-binding proteins, RalA and RalB, first surfaced over 20 years ago when Pierre Chardin isolated their cognate genes from a hybridization screen of B-lymphocyte cDNAs using degenerate probes containing highly conserved Ras sequence. With 82% identity to each other at the amino-acid level, we now know that RalA and RalB represent the inclusive roster for the Ral branch of the over 170-strong Ras family G-protein tree. Importantly, both gain-of-function and loss-of-function studies identified Ral activation as a proximal consequence of Ras expression that could contribute to Ras-induced oncogenic transformation in cell culture model systems. Ral G-proteins are activated to a GTP-bound state in a variety of human tumors. In this "active" state, they are able to bind to a number of effector molecules, which regulate diverse biological processes such as cytokinesis, endocytosis, and exocytosis. RNA interference-mediated depletion of the individual isoforms revealed divergent signaling roles for RalA and RalB, with RalA promoting anchorage-independent growth and RalB as a linchpin molecule supporting cancer cell survival. Recently, we uncovered a RalB-specific contribution to cancer cell survival through activation of TANK-binding-kinase 1 (TBK1). This kinase is a central node in the regulatory network required to trigger host defense gene expression in the face of a virally compromised environment. Through a process that is tethered to the exocyst but perhaps independent of canonical exocyst function, the RalB-Sec5 effector complex directly recruits and activates TBK1 in response to viral exposure. Although dispensable for survival of normal cells in culture, constitutive engagement of this pathway in a variety of cancer cells, through chronic RalB activation, restricts initiation of apoptotic programs that are normally activated in the context of oncogenic stress. To further characterize the role of Ral G-proteins in host surveillance, we investigated their importance in macroautophagy. Macroautophagy, herein referred to simply as autophagy, is a process by which a cell catabolizes and degrades cytoplasmic materials, dysfunctional organelles, or foreign invaders. Autophagy has been implicated as an important process in both innate and adaptive immune responses. The role of autophagy in cancer is enigmatic as it has been implicated as a tumor suppressor pathway but confusingly is chronically upregulated in many tumor-derived cell lines. A peptide inhibitor of both Ral G-proteins was able to block both baseline autophagosome formation and autophagy stimulated by infection with a foreign invader, Salmonella. To determine isoform specificity of Ral signaling in autophagy, we depleted both isoforms using RNA interference, and depletion of RalB but not RalA from endometrial cancer cells led to a marked reduction in the baseline formation of autophagosomes. Depletion of a core component of the

exocyst complex, Sec8, also led to a marked reduction in baseline autophagosome formation indicating the exocyst may be the Ral effector pathway utilized by RalB in autophagosome formation. Future studies will be aimed to (1) further delineate the mechanistic link between RalB activation and autophagosome formation, (2) understand the role of autophagy in the overall contribution of RalB to innate and adaptive immune signaling, and (3) understand how cancer cells subvert this machinery to their advantage.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0749 and National Institutes of Health.

P65-9: TARGETING Rheb SIGNALING AND POSTTRANSLATIONAL PROCESSING FOR THE TREATMENT OF BREAST CANCER

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Background and Objectives: Rheb is a critical component of the Akt-TSC-mTOR pathway that regulates protein translation, nutrient sensing, cell size, and cell proliferation. Recent studies found that estrogen stimulation of estrogen receptor-positive MCF-7 breast cancer cells causes Rheb activation, leading to mTOR-mediated phosphorylation of S6K. Additionally, Rheb was shown to be required for estrogen-induced DNA synthesis and cell cycle progression in MCF-7 cells. However, whether Rheb is required for estrogen-mediated proliferation and tumorigenesis is not yet known. Rheb is targeted to endomembranes via its C-terminal CAAX tetrapeptide motif, a substrate for posttranslational modification by a farnesyl isoprenoid lipid. A series of further CAAX-signaled posttranslational processing, including -AAX cleavage (catalyzed by Rce1) and carboxyl methylation (catalyzed by Icm1), are required for Rheb localization. Farnesylation and proper localization are critical for Rheb function. Our work addresses the possibility that Rheb is a clinically relevant target for farnesyltransferase (FTase) inhibitors (FTIs). FTIs were developed originally to inhibit Ras processing and membrane localization. While FTIs have shown anti-tumor activity in clinical trials, this activity is not due to inhibition of Ras but to inhibition of other FTase substrates. FTIs were shown previously to potently inhibit the growth of MCF-7 cells, but whether the sensitivity of MCF-7 cells to FTIs is due to Rheb inhibition is not known. The objectives of our research are to validate Rheb as a target for breast cancer treatment and to determine if blocking Rheb posttranslational processing and subsequent membrane association inhibits breast cancer growth.

Methods: To validate Rheb as a target for breast cancer treatment, we developed interfering shRNA to stably repress Rheb expression in MCF-7 cells and analyzed the tumorigenic properties of these cells. We also stably transfected MCF-7 cells with an FTI-insensitive Rheb variant, geranylgeranylated Rheb (GG-Rheb), to determine if the anti-tumor activity of FTIs is due to Rheb inhibition. Finally, we will use mouse embryonic fibroblasts lacking Icm1 or Rce1 to determine if other steps of Rheb posttranslational processing are required for Rheb function.

Results to Date: We have confirmed that knocking down Rheb expression decreased phosphorylation of S6K and the ribosomal S6 subunit, downstream targets of the Rheb effector mTOR. We found that knocking down Rheb expression slightly decreased the anchorage-independent growth of MCF-7 cells but did not significantly decrease long-term estrogen-stimulated growth, suggesting that Rheb may not be a critical mediator of estrogen-induced breast cancer growth. Finally, we verified that GG-Rheb is insensitive to FTI inhibition and preliminary analyses indicate that it did not reduce MCF-7 sensitivity to FTI treatment.

Conclusions and Impact: The results of these studies will determine whether Rheb is a valid therapeutic target for breast cancers. Furthermore, these experiments will determine whether the anti-tumor effects of FTIs in breast cancer depend on Rheb. Since FTIs are already in clinical trials for the treatment of breast cancer, these studies could have significant implications for the selection of patients that may benefit from FTIs.

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P65-10: VITAMIN D RECEPTOR ENHANCES TCF7L2 EXPRESSION, WHICH MAY SUBSEQUENTLY INHIBIT WNT-REGULATED GENES IN THE BREAST

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Since the discovery that people living in cities and northern latitudes have less circulating vitamin D and a higher incidence of breast cancer, epidemiology, animal models, and molecular studies have supported vitamin D's role as an anti-cancer agent. However, use of vitamin D and its analogues is limited by its tendency to cause hypercalcaemia, a potentially life-threatening condition. It is critical, therefore, to discover what molecules convey vitamin D's anti-cancer properties so they can be exploited as drug therapy targets while those molecules that mediate vitamin D's calcium effects can be avoided.

It has been shown that vitamin D imparts its anti-cancer properties through the vitamin D receptor (VDR). In order to discover new, potential mechanisms by which vitamin D can mediate its effects, we have been utilizing a set of transformed cell lines that are derived from DMBA-induced mouse mammary tumors from mice that were null or wild type for the VDR (VDR $-/-$ and VDR $+/-$, respectively). We observed that the VDR $+/-$ cells had far greater expression of TCF7L2 with respect to the $-/-$ cells. TCF7L2 (aka TCF4) is a major player in the Wnt pathway, which has been shown to be dysregulated in numerous cancers, including breast. The Wnt pathway's most studied member is β -catenin, a multifunctional protein that is involved in cell adhesion and transcriptional co-activation with the TCF/Lef family of transcription factors. In response to the presence of canonical Wnts, β -catenin is protected from proteasomal degradation and accumulates in the cytoplasm. It is then available to translocate to the nucleus and coactivate transcription of TCF-bound genes involved in cellular transformation. In the absence of activated β -catenin, TCF7L2 has been shown to act as a repressor. Activated β -catenin is not typically overexpressed in malignant or normal breast tissue; however, it is expressed during certain developmental stages. We have shown that exogenous VDR increases TCF7L2 expression in VDR $-/-$ cells. We have also shown that treatment with vitamin D can increase expression of TCF7L2 in some cell lines. We also show that a luciferase construct containing 5' upstream regions of the TCF7L2 gene shows a trending increase in expression in mammary cells when treated with 1,25(OH) $_2$ D $_3$ in the presence of VDR. Last, we are able to show that treatment of CaCo2 cells with 1,25(OH) $_2$ D $_3$ enhances expression of the TopFlash reporter, a β -catenin /TCF7L2-regulated construct. Knockdown of VDR abolishes 1,25(OH) $_2$ D $_3$ induction of the TopFlash reporter. These findings, thus far, are in support of our hypothesis that the VDR upregulates TCF7L2 levels and that this may inhibit Wnt-regulated genes in the breast.

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P65-11: ROLE OF THE p97 AAA-ATPase IN LIGAND-DEPENDENT NUCLEAR LOCALIZATION OF EPIDERMAL GROWTH FACTOR RECEPTOR

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Trafficking of mature EGF receptor has traditionally been limited to sites of post-endosomal targeting such as the lysosome. However, it is now known that mature receptor can traffic to the nucleus and affect gene transcription (*Nat. Cell. Biol.* 9:802-808 (2001)). Until recently, the mechanism of intact EGF receptor trafficking to the nucleus was not clear and little was known about which proteins supported this process. Recent data by Liao and Carpenter (*MCB* 18:1064-1072 (2007)) defines a novel requirement of the Sec61 translocon in ligand-dependent retrotranslocation and subsequent nuclear localization of the EGF receptor. The mechanism of dislocation of mature EGF receptor from the Sec61 translocon is unclear. The AAA-ATPase p97 is known to participate in dislocation of ERAD substrates from the Sec61 translocon (*Nature* 414:652-656 (2001)). My preliminary data demonstrates ligand-dependent coprecipitation of EGF receptor and p97 at a time point consistent with that of ligand-dependent localization of the EGF receptor to the Sec61 translocon. In addition, I have utilized RNA interference to knock down p97 to demonstrate its requirement for nuclear localization of the EGF receptor.

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P65-12: ROLE OF Stat5 IN HUMAN BREAST CANCER

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Stat5 is a transcription factor activated by the prolactin (PRL)-Jak2 pathway and is necessary in mammary gland differentiation and lactation. Despite reports of a tumorigenic role in breast cancer, recent evidence suggests that active Stat5 may have a tumor suppressive role in breast cancer progression. We have previously shown that active Stat5 is a favorable prognostic marker in breast cancer patients and is lost with increasing metastatic progression. Further, Stat5 was able to induce differentiation markers and suppress invasive characteristics in breast cancer cell lines. We propose that active Stat5 is able to induce differentiation which serves as a means to suppress invasion and metastasis of breast cancer cell lines. To further investigate the role of active Stat5 in human breast cancer, we designed two constitutively active Stat5a constructs, Stat5-S710F and Stat5-3ser, which are tyrosine phosphorylated and transcriptionally active in the absence of prolactin stimulation. We hypothesize that over expression of active Stat5 will correlate with increased expression of differentiation markers and reduced invasion in vitro and in vivo. Stat5-S710F was generated by a point mutation of serine 710 to phenylalanine. Stat5-3ser contains the S710F mutation in addition to mutations of serine 725 and serine 779 to alanines. Preliminary studies have indicated that Stat5a-3ser has a greater potential to be active in the absence of prolactin stimulation. We have generated lentiviral, adenoviral, and T47D and MDA-MB-231 stable cell lines expressing these constructs and are in the process of initiating both in vitro and in vivo

differentiation and invasion studies. These constructs will allow us to investigate the role of Stat5 in breast cancer progression separate independent of other PRL-induced downstream signals.

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P65-13: ACTIVATED Akt1 ACCELERATES MMTV-c-ErbB2 MAMMARY TUMORIGENESIS IN MICE WITHOUT ACTIVATION OF ErbB3

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ErbB2, a member of the epidermal growth factor receptor family (EGFR), is overexpressed in 20%–30% of human breast cancer cases and forms oncogenic signaling complexes when dimerized to ErbB3 or other EGFR family members. We have crossed the MMTV-myr-Akt1 transgenic mice (which express constitutively active Akt1 in the mammary gland) with MMTV-c-ErbB2 transgenic mice to evaluate the role of Akt1 activation in ErbB2-induced mammary carcinoma. Bitransgenic MMTV-c-ErbB2, MMTV-myr-Akt1 mice develop mammary tumors twice as quickly as the MMTV-c-ErbB2 mice. Histology and activated caspase 3 immunohistochemistry demonstrate that the bitransgenic tumors are more necrotic, less organized, and less apoptotic than the tumors from MMTV-c-ErbB2 mice.

The two tumor types demonstrate dramatically different expression and activation of EGFR family members as well as different metabolic profiles. c-ErbB2 tumors demonstrate overexpression of EGFR, ErbB2, ErbB3, and ErbB4 and activation of ErbB2 and ErbB3, underscoring the importance of the entire EGFR family in ErbB2-induced tumorigenesis. Tumors from bitransgenic mice demonstrate overexpression of the myr-Akt1 and ErbB2 transgenes; however, there was dramatically less overexpression and phosphorylation of ErbB3, decreased levels of EGFR and ErbB4 proteins, decreased ErbB2 phosphorylation, and decreased downstream tyrosine phosphorylation as compared to the c-ErbB2 tumors. Thus, activation of Akt1 alters the requirement for EGFR family signaling in c-ErbB2-induced tumorigenesis. The bitransgenic tumors contain more lactate as well as the glucose transporter, GLUT1, suggesting increased glucose transport and glycolysis. However, GLUT1 appears to be induced by hypoxia rather than by activation of Akt.

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P65-14: ABSTRACT MOVED TO P30-28.

P65-15: A NOVEL ROLE FOR p115 RhoGEF AND LARG IN FIBRONECTIN-INDUCED RhoA ACTIVATION

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Background and Objectives: The chances of survival for a patient with breast cancer are dramatically reduced when tumor cells transition from a primary malignant to an invasive, metastatic state. The ability of a tumor cell to efficiently execute the steps of metastasis—invading through the surrounding extracellular matrix (ECM) to the blood stream, followed by extravasation at distant sites—is directly linked to its invasive and migratory properties. The mechanisms involved in normal cell migration in response to ECM adhesion have been partly characterized. When a cell adheres to ECM substrates such as fibronectin (FN), adhesion molecules such as integrins signal to the different members of the Rho family of small GTPases, the prototypical family members being RhoA, Rac1, and Cdc42. Rho GTPase regulation is controlled by guanine nucleotide exchange factors (GEFs, which activate GTPases by exchanging GDP for GTP) and GTPase activating proteins (GAPs, which inactivate GTPases by increasing GTP hydrolysis rates).

Considering the importance of Rho GTPases in the metastatic process, it is surprising that no activating mutations for these proteins have been found in cancer, unlike Ras GTPases. In particular, no mutations have been found for RhoA in invasive breast cancers. However, mutations for several GEFs (Bcr, LARG, and Tiam1) have been found in different types of cancer. The current study is therefore focused at understanding the roles of GEFs in the migratory process of metastatic breast cancer cells (BCCs) in response to ECM adhesion.

Methodologies and Results: Using an affinity pulldown for activated exchange factors, we show that the RhoA-specific exchange factors Lsc/p115 RhoGEF and LARG are activated when fibroblasts are plated onto FN. This effect is specific to Lsc/p115 RhoGEF and LARG, as other exchange factors such as Ect2 or Dbl are not activated by FN. Knockdown of Lsc and LARG together significantly decreases RhoA activation, stress fiber, and focal adhesion formation downstream of fibronectin adhesion. Identical

results on RhoA signaling are obtained with a catalytically inactive mutant of p115 RhoGEF, suggesting that catalytic activity of the GEF is necessary for downstream signaling to RhoA. p115 RhoGEF and LARG are part of a family of RhoA GEFs known as RGS-GEFs, which are traditionally known for their involvement in serum-induced RhoA activation. Interestingly, we observe that while G-protein coupled receptors are not involved in FN-induced p115 RhoGEF activation, integrin adhesion is sufficient to activate p115 RhoGEF.

Conclusions and Significance: These data therefore establish a previously uncharacterized role for the exchange factors Lsc/p115 RhoGEF and LARG in linking fibronectin signals to downstream RhoA activation. Further studies will include analysis of the roles of these RGS-GEFs in RhoA activation and migratory and metastatic properties of BCCs. By investigating the role of GEFs in breast cancer metastasis, the current study will fill a significant gap in our understanding of Rho protein signaling in cancer cell motility and provide insights that may lead to the design of novel therapeutic strategies to counter breast cancer metastasis.

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P65-16: NONLINEAR IMAGING OF Rho ACTIVITY IN BREAST CANCER CELLULAR MODELS

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Appropriate extracellular matrix (ECM) tissue environments regulate the polarization of breast epithelial cells into ductal structures and are necessary for the development of a functional mammary gland. Increased breast density resulting from enhanced collagen deposition can result in disruption of normal ductal morphology and eventually carcinoma. At the cellular level, the physical alterations of the surrounding ECM have been shown to be important regulators of cellular differentiation, proliferation, and gene transcription. Cells interact with ECM components through trans-membrane receptors, such as integrins, to activate mechano-signaling events that regulate this differentiation and proliferation. However, signaling events that occur in relevant three-dimensional matrices can be difficult to study using conventional biochemical approaches. To help better understand the temporal and spatial regulation of complex signaling events within relevant matrices, our group has developed multidimensional Multiphoton Laser Scanning Microscopy (MPLSM) signal acquisition techniques. This technology enables collection of fluorescence intensity, spectral, lifetime, and second harmonic (SHG) signals simultaneously from deep within live cell and tissue samples. In this study, high resolution imaging of matrix and signaling components were used to better elucidate the interrelation between matrix attachment, cell proliferation, and motility in breast epithelial models. T47D human breast cancer cells were stably transfected with fluorescently tagged Rho and the Rho binding domain (RBD) of roteklin. The cells were then cultured within collagen matrices and imaged at timepoints through breast tubule formation. Analysis of these multidimensional signals are used to map regions of intercellular protein localization and environment to understand their interaction with the extracellular matrix through time.

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P65-17: ALTERED EGFR LOCALIZATION AND DEGRADATION IN HUMAN BREAST CANCER CELLS WITH AN AMPHIREGULIN AUTOCRINE LOOP

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The epidermal growth factor receptor (EGFR) and its ligand amphiregulin (AR) have been shown to be co-overexpressed in breast cancer. We have previously shown that an AR/EGFR autocrine loop is required for SUM149 human breast cancer cell proliferation, motility, and invasion. We also demonstrated that AR can induce these altered phenotypes when expressed in the normal mammary epithelial cell line MCF10A, or by exposure of these cells to AR in the media. In the present studies, we demonstrate that SUM149 cells and immortalized human mammary epithelial MCF10A cells that overexpress AR (MCF10A AR) or are cultured in the presence of exogenous AR, express higher levels of EGFR protein compared with MCF10A cells cultured in EGF. Pulse chase analysis showed that EGFR protein remained stable in the presence of AR yet was degraded in the presence of EGF. Consistent with this observation, tyrosine 1045 on the EGFR, the c-cbl binding site, exhibited decreased phosphorylation in the presence of AR compared with EGF. Ubiquitination of the receptor was also dramatically decreased when AR was the ligand as compared with EGF. Flow cytometry analysis showed that EGFR remained on the cell surface following AR activation instead of being rapidly internalized as observed when EGF was present. Immunofluorescence and confocal microscopy confirmed the flow cytometry results. MCF10A cells cultured in the presence of EGF exhibited a predominantly intracellular, punctate localization. In stark contrast, SUM149 cells and MCF10A cells growing in the presence of AR expressed EGFR predominantly on the membrane and at cell-cell junctions. Based on these data, we propose that AR alters EGFR internalization and degradation in a way

that favors accumulation of EGFR at the cell surface and ultimately leads to changes in EGFR signaling. This change in EGFR signaling can have dramatic effects on breast tumor growth and aggressiveness. Therefore, AR may be an effective target in the treatment of breast cancer.

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P16-18: PHOSPHOLIPASE C γ 1 REGULATES FIBRONECTIN ASSEMBLY

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Cell adhesion and migration are critical for cancer cells metastasis. Many signaling pathways are involved in adhesion and migration. This includes the growth factor receptor tyrosine kinase and integrin signaling pathways. Phospholipase C γ 1 (PLC- γ 1) is an enzyme that is activated downstream of both of these pathways. Phosphorylation of PLC- γ 1 by tyrosine kinases such as the epidermal growth factor receptor and Src activate the enzyme and result in the hydrolysis of phosphoinositol 4,5, bisphosphate to form inositol 1,4,5 trisphosphate and diacylglycerol. These second messengers facilitate the release of calcium from the endoplasmic reticulum and activation of protein kinase C, respectively. The downstream effects of PLC- γ 1 have yet to be fully elucidated. Absence of PLC- γ 1 results in reduced migration but the mechanism is not yet understood. My research aims to determine the mechanism by which PLC- γ 1 regulates adhesion and migration. Studies using mouse embryonic fibroblasts null for PLC- γ 1 (Null) and add back cells (Null +) indicate that PLC- γ 1 negatively regulates the assembly of fibronectin fibrils. In hanging drop assays, Null cells form significantly tighter aggregates than Null + cells as measured by resistance to disaggregation by pipetting. As fibroblasts do not form cell-cell contacts, I hypothesized that the cells adhere to an extracellular matrix component that is either increased or selectively produced in the Null cells. Deoxycholic acid solubility assays revealed that Null cells were indeed assembling more endogenous fibronectin matrix than Null + cells. This increased assembly is not due to increased fibronectin mRNA nor is it due to increased integrin expression in the Null cells. To further distinguish assembly from protein levels and secretion, exogenous assembly assays were performed. Once again, Null cells assemble more fibronectin than Null + cells. These results indicate that PLC- γ 1 may negatively regulate the assembly of fibronectin and extracellular matrix composition. The increase in fibronectin assembly therefore, may account for the increased aggregation and reduced migration observed in mouse embryonic fibroblasts lacking PLC- γ 1.

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P65-19: CHARACTERIZATION OF Odin, A NOVEL INHIBITORY MOLECULE, IN EGF RECEPTOR SIGNALING

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Receptor tyrosine kinase (RTK) signaling plays an important role in a variety of cellular processes and is tightly controlled by various positive and negative regulatory mechanisms. Dysregulation of RTK signaling contributes to the development of cancers, and understanding the regulation of RTK signaling can potentially help the development of new therapeutic methods. Odin, a novel adaptor protein, has been shown to play an inhibitory role in the RTK signaling pathway; however, the underlying mechanism by which Odin regulates RTK signaling remains unknown. The goal of our proposal is to dissect the function of Odin in the RTK signaling pathway and its regulation in this process.

As an adaptor protein, Odin contains six ankyrin repeats, two sterile alpha motifs, and one phosphotyrosine-binding domain, and we reasoned that Odin regulates RTK signaling through interacting with other signaling molecules. So we sought to identify the interacting proteins of Odin using a quantitative mass spectrometry-based approach using the SILAC method, which can distinguish true binding proteins of Odin from nonspecific binding proteins to antibodies/protein G/agarose matrix. Of more than 250 identified proteins, we identified 16 proteins as potential interactors of Odin. All seven members of 14-3-3 adaptor proteins have been identified as Odin interacting partners although the previous data have shown Odin in a protein complex of 14-3-3 gamma and sigma. Three interacting proteins of Odin, RASAL2, DAB2IP, and ARHGAP10, are from the GTPase activating protein family, of which RASAL2 and ARHGAP10 were also reported as interacting proteins of 14-3-3 proteins. CD2AP and SH3KBP1 both contain three SH3 domains and have been proposed to play an important role in the downregulation of RTK through promoting endocytosis of RTK after ligands stimulation. Other interacting proteins that were identified include CAPZB, TALIN2, VAPA, UACA, GART, and HSPA9B. We have validated the interaction between Odin and 14-3-3 proteins, CD2AP, SH3KBP1, and Talin2, using co-immunoprecipitation. Since several proteins are involved in endocytosis, we will examine the role of Odin in the process of receptor endocytosis after ligand stimulation.

In addition, Odin undergoes tyrosine phosphorylation after ligand stimulation; however, the phosphorylation sites and the upstream kinase are unknown. So we performed a peptide array-based in vitro kinase assay and identified two novel tyrosine phosphorylation sites (Tyr 361 and Tyr 455) by c-Src. These data also implicate Odin in the downstream of Src in the RTK signaling pathway. Interestingly, we also identified two phosphorylation sites on serine residues (Ser 647 and Ser 663) using mass spectrometry in the above experiment, which have also been reported recently by others. The identification of phosphorylation sites will allow us to study how phosphorylation regulates the function of Odin.

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P65-20: DIFFERENTIAL EFFECTS OF Stat3 INHIBITION IN SPARSE VERSUS CONFLUENT NORMAL AND BREAST CANCER CELLS

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Background and Objectives: The signal transducer and activator of transcription-3 (Stat3) is hyperactive in breast cancers and is required for transformation by a number of oncogenes. Signaling through Stat3 is determined by a key phosphorylation at tyr-705. We previously demonstrated that cell to cell adhesion brought about through cell aggregation or confluence of cultured cells causes a dramatic increase in Stat3 activation and consequently Stat3 activity in both normal and tumor cells.

Methodologies: To examine the role of Stat3 at specific timepoints relative to confluence, we used two different approaches of Stat3 inhibition: (1) Introduction of high levels of peptides or peptide analogs, which block the Stat3-SH2 domain, to inhibit Stat3 binding to and phosphorylation by growth factor receptors. Since these molecules do not cross the cell membrane, we developed a technique of electroporation of adherent cells in situ as the cells grow on a slide that is coated with conductive and transparent Indium-Tin oxide. This turned out to be a powerful technique for signal transduction studies, and it was modified to examine gap junctional communication (Anagnostopoulou et al., *Molecular Oncology* 1:226-231). (2) Treatment with two platinum compounds that bind Stat3 and inhibit its activity without affecting its phosphorylation directly. In collaboration with Dr. Baird, an inorganic chemist, we developed a technique to synthesize CPA7, a very effective Stat3 inhibitor (Littlefield, Baird, Anagnostopoulou, Raptis, *Inorganic Chemistry*, in press).

Results: The results demonstrate that Stat3 downregulation in vSrc transformed NIH3T3 cells, cells transformed by the Simian Virus 40 Large tumor antigen, an inhibitor of Rb that is often inactivated in breast cancer, or in breast cancer lines harboring activated Src induces apoptosis, which is evident at all densities but is more pronounced at post-confluence. In normal cells, on the other hand, Stat3 inhibition at post-confluence caused apoptosis while in sparsely growing cells it induced merely a growth retardation (Anagnostopoulou et al., *Cancer Letters*, 242:120; Anagnostopoulou et al., *Trends in Cancer Research*, 2:93-103, 2007).

Examination of the effect of Stat3 inhibition upon levels of p53, a Stat3 target gene, at different cell densities indicated a dramatic increase in p53 levels. This could be responsible for the induction of apoptosis following Stat3 inhibition since cells devoid from, or expressing mutant p53, were found to be resistant to Stat3 inhibition.

We will use the technique we developed to examine gap junctional, intercellular communication in cells expressing constitutively active Stat3 or following Stat3 downregulation using CPA7.

Conclusions: Our findings point to the possibility that in cultured cells and potentially in vivo, Stat3 inhibition may be a very effective way to induce apoptosis specifically in tumor cells. This is especially important for breast cancers where Herceptin and many other tyrosine kinase inhibitors are ineffective since the cell density-dependent, Stat3 activation was found to be independent from membrane tyrosine kinases such as ErbB2, IGF1R, and the Src family. Challenging the existing thesis, this work holds great promise in anti-cancer drug design once the relevant tumors are identified.

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P65-21: p130Cas AND BCAR3-MEDIATED REGULATION OF c-Src KINASE ACTIVITY

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Estrogen receptor-positive breast cancer cells can become resistant to antiestrogen treatment through overexpression of either the adaptor protein p130Cas (Cas; also known as breast cancer antiestrogen resistance-1, BCAR1) or BCAR3 (breast cancer antiestrogen resistance-3; also known as murine AND-34). Coincidentally, increased expression and activation of the nonreceptor tyrosine kinase, c-Src, has been implicated

in the development, growth, and metastasis of breast tumors. Previous work from our lab showed that overexpression of Cas increased c-Src kinase activity and substrate phosphorylation and that co-overexpression with BCAR3 augmented c-Src kinase activation above that found with Cas overexpression alone. We also showed that Cas-induced antiestrogen resistance occurs through a pathway that requires c-Src. The focus of the current study has been to identify mechanisms by which co-overexpression of Cas and BCAR3 regulates c-Src activity in breast cancer cells, thus potentially leading to antiestrogen resistance. We have found that activation of c-Src in Cas/BCAR3-overexpressing cells requires Cas-Src interactions, the small GTPase Rap1, and the Cas binding partner, focal adhesion kinase (FAK). Many breast tumor cell lines, including BT549 and MDA-MB-231 cells, overexpress Cas and BCAR3. Thus, they serve as excellent model systems with which to dissect Cas and BCAR3-mediated regulation of c-Src activity, substrate phosphorylation, and cell survival, proliferation, and migration. Preliminary studies using BT549 cells show that depletion of BCAR3 from cells results in decreased levels of activated c-Src following adhesion to fibronectin. We are currently seeking to identify the mechanisms by which c-Src activity becomes reduced under conditions of BCAR3 depletion and the biological consequences of these events. By characterizing the mechanism(s) of c-Src regulation by Cas and BCAR3, we hope to expand upon our current understanding of how c-Src kinase activity is upregulated in breast cancer cells. Because this pathway appears to be functionally linked to antiestrogen resistance, this work will also aid in the development of better therapeutic approaches for patients whose tumors overexpress both Cas and BCAR3 and do not respond to antiestrogens such as tamoxifen.

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P65-22: OVEREXPRESSION OF EphA2 RECEPTOR DESTABILIZES ADHERENS JUNCTIONS VIA A RHOA-DEPENDENT MECHANISM

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A new family of receptor tyrosine kinases, the Eph family, plays a critical role in cancer. Originally discovered as modulators of axonal guidance and embryonic patterning during development, subsequent studies have shown that many Eph receptors are overexpressed in a large number of cancers (Brantley-Sieders et al., 2004; Pasquale, 2005). One family member in particular, the EphA2 receptor, has been linked to breast cancer, prostate cancer, lung cancer, ovarian and cervical cancers, esophageal and colorectal cancers, as well as malignant melanoma (Iretton and Chen, 2005). Furthermore, the level of EphA2 receptor expressed on tumor cells correlates with the degree of tumor malignancy (Kinch and Carles-Kinch, 2003). Overexpression of the EphA2 receptor in MCF-10A cells is associated with increased cell growth in soft agar and increased tumor growth when these cells were implanted into nude mice (Zelinski et al., 2001). However, despite the strong correlation of EphA2 receptor expression with malignant phenotypes, the mechanisms by which EphA2 contributes to tumor cell malignancy are not completely understood. Here we report that overexpression of a wild-type EphA2, but not a signaling defective cytoplasmic truncation mutant (ΔC), in human mammary epithelial cells weakens cell-cell adhesion. Interestingly, the total levels of cadherins and the composition of the adherens junction complexes were not affected nor was the tyrosine phosphorylation of the cadherin complex components changed. In contrast, RhoA GTPase activity was significantly affected by modulating the EphA2 activity in MCF10A cells. Treatment with a ROCK kinase inhibitor rescued cell-cell adhesion defects in EphA2-overexpressing cells, whereas expression of constitutively activated Rho disrupted adherens junctions in ΔC -overexpressing cells. EphA2-dependent Rho activation and destabilization of adherens junctions appeared to be regulated via a signaling pathway involving Src kinase, LMW-PTP, and p190 RhoGAP. EphA2 interacted with both Src and LMW-PTP, and the interactions increased in EphA2-overexpressing cells. In addition, LMW-PTP phosphatase activity was elevated, and this elevation was accompanied by a decrease in tyrosine phosphorylation of p190 RhoGAP and destabilization of cell-cell adhesion. Expression of either a dominant-negative LMW-PTP mutant, C12S, or wild-type p190 RhoGAP rescued adhesion defects in EphA2-overexpressing cells. Together, these data suggest that EphA2 promotes tumor malignancy through a mechanism involving RhoA-dependent destabilization of cell-cell adhesion. These findings suggest EphA2 may be a novel therapeutic target in breast cancer.

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P65-23: PHLPP: A NOVEL FAMILY OF PHOSPHATASES THAT ARE CRITICAL REGULATORS OF Akt AND PKC SIGNALING AND PLAY A POTENTIAL ROLE IN BREAST CANCER

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A fundamental aim in cell signaling is to decipher how phosphorylation and dephosphorylation regulate cell fate. In order to achieve this goal, the scientific community must have a complete understanding of the proteins involved in regulating this complex cellular process. To this end, we have discovered a novel family of phosphatases

referred to as PHLPP, (PH domain leucine-rich repeat protein phosphatase). The Ser/Thr specific-phosphatase PHLPP provides the brakes to signaling by Akt and protein kinase C (PKC). The two isoforms of this recently discovered family, PHLPP1 and PHLPP2, control the amplitude and duration of signaling of Akt and PKC by catalyzing the dephosphorylation of the hydrophobic phosphorylation motif, a site that controls the activity of these kinases. Aberrant regulation of either kinase accompanies many diseases, notably cancer. By specifically dephosphorylating the hydrophobic motif, PHLPP controls the degree of agonist-evoked signaling by Akt and the cellular levels of PKC. Although PHLPP1 and PHLPP2 both dephosphorylate the same residue (hydrophobic phosphorylation motif) on Akt, they differentially terminate Akt signaling by regulating distinct Akt isoforms. Knock-down studies reveal that PHLPP1 specifically modulates the phosphorylation of HDM2 and GSK-3 α through Akt 2, whereas PHLPP2 specifically modulates the phosphorylation of p27 through Akt 3. These data unveil a new mechanism to selectively terminate Akt signaling pathways through the differential inactivation of specific Akt isoforms by specific PHLPP isoforms.

PHLPP2 is poised to be a tumor suppressor based on its negative regulation of Akt as well its chromosomal location at 16q22.3, a region that encounters frequent loss of heterozygosity in breast cancer. While sequencing for somatic mutations in breast cancer we discovered a novel polymorphism resulting in an amino acid change from a Leu to Ser at codon 1016 in the phosphatase domain of PHLPP2. Analysis of HA-PHLPP2 constructs with either a Ser or Leu at position 1016 revealed the Ser1016 variant has reduced phosphatase activity toward phosphorylated Akt both in vitro and in cells, as well as an attenuated apoptotic effect. Depletion of endogenous PHLPP2 variants in breast cancer cells revealed the Ser1016 variant is less functional toward both Akt and PKC. In pair-matched, high-grade breast cancer samples we observe retention of only the Ser allele from heterozygous patients (identical results were observed in a pair-matched normal and tumor cell line). In conclusion, we have identified a functional polymorphism that impairs the activity of PHLPP2 and potentially plays a role in breast tumorigenesis.

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P65-24: THE ROLE OF p55 γ IN PHOSPHATIDYLINOSITOL 3-KINASE FUNCTION

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Class IA PI3Ks are regarded as the most important in regulating cell proliferation and tumorigenesis. The p55 γ protein is a regulatory subunit of class IA PI3K. In vitro study has demonstrated that the NH2-terminal of p55 γ is sufficient to bind the cell cycle regulatory protein pRb. Association between calmodulin and p55 γ in 293T cells has been demonstrated by calmodulin sepharose beads pull-down assay in the previous report. We also demonstrated that p55 γ stabilized the interaction between calmodulin and Rb. We aim to uncover the cell cycle and growth regulation effect of p55 γ protein by overexpression and RNA interference analysis. Here we confirmed the co-immunoprecipitation of Rb with p55 γ protein. However, by overexpression of full-length p55 γ in MCF-7 cells or knocking down of p55 γ in AU565 cells, we could not demonstrate a significant effect on the cell cycle or cell growth. Using SK-Br-3 cells as a model for breast cancer cells, we found a shift in composition among the PI3K regulatory subunits once p55 γ was manipulated. Other PI3K regulatory subunits such as p85 α and p50 α increased when p55 γ was knocked down, and p85 α decreased when p55 γ was overexpressed. This could account for the marginal effect we observed when we expressed or down-regulated p55 γ in breast cancer model systems.

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P65-25: FAK POTENTIATES Rac1 ACTIVATION AND LOCALIZATION TO MATRIX ADHESION SITES: A ROLE FOR β -PIX

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Rac and FAK are involved in the regulation of proliferation, growth, and metastasis in breast cancer cells. FAK, a cytoplasmic protein tyrosine kinase, is activated and localized to focal adhesions upon cell attachment to extracellular matrix. FAK null cells spread poorly and exhibit altered focal adhesion turnover. Rac1 is a member of the Rho-family GTPases that promotes membrane ruffling, leading edge extension, and cell spreading. We investigated the activation and subcellular location of Rac1 in FAK null and FAK re-expressing fibroblasts. FAK re-expressors had a more robust pattern of Rac1 activation following cell adhesion to fibronectin than the FAK null cells. Translocation of Rac1 to focal adhesions was observed in FAK re-expressors but seldom in FAK null cells. Experiments with constitutively active L61Rac1 and dominant-negative N17Rac1 indicated that the activation state of Rac1 regulated its localization to focal adhesions. We demonstrated that FAK tyrosine-phosphorylated β PIX and thereby increased its binding to Rac1. In addition, β PIX facilitated the targeting of activated Rac1 to focal adhesions and the efficiency of cell spreading. These data indicate that

FAK has a role in the activation and focal adhesion translocation of Rac1 through the tyrosine phosphorylation of β PIX. This represents a novel mechanism of Rac regulation and may provide an effective drug targeting strategy for agents that could slow tumor growth and limit metastatic potential.

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P65-26: INHIBITION OF ADAPTER TYPE MOLECULE p130Cas SIGNALING IN BREAST CANCER

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Aberrant expression of the epithelial growth factor receptor (EGFR) tyrosine kinase family and the non-receptor focal adhesion kinase (FAK) has been implicated in the genesis of a significant proportion of sporadic human breast cancers. EGFR and Her2/neu are overexpressed in about 48%, and 20%–30% of all human breast cancers, respectively. Overexpression of FAK has been reported in 88% of breast cancer samples, and elevated FAK levels are frequently associated with advanced disease. The p130Cas-Crk complex, which forms in response to activation of those kinases, has been proposed as a central molecular switch that mediates the communication between upstream extracellular signals and downstream intracellular events. Thus, the development of novel approaches to block p130Cas activation and signaling may prove useful as anti-cancer therapies. Here we present data using two peptide approaches. 1. A novel fusion protein with the substrate domain (SD) of p130Cas (CasSD), which functions as a decoy molecule, and thereby acts as dominant negative (DN) of p130Cas signaling. To investigate the effects of CasSD in vitro, breast cancer cell lines were stably infected. CasSD expression reduced migratory behavior and resulted in redistribution of the cell-cell adhesion molecule E-cadherin to the cell membrane. Thus, inhibition of p130Cas attenuates epithelial-to-mesenchymal transition (EMT), suggesting that elevated signaling through the p130 Cas SD signaling node contributes to breast cancer cell invasive phenotype. 2. The pro-peptide region (LOX-PP) of lysyl oxidase, an extracellular enzyme critical for the biosynthesis of extracellular matrix proteins. The LOX gene product (Pro-LOX) was found to inhibit Ras-mediated transformation of NIH 3T3 cells. Recently, our group demonstrated that the tumor suppressor is attributed to the LOX-PP. LOX-PP was found to be a potent inhibitor of breast cancer cells driven by the Her-2/neu receptor, which signals via Ras. Here we explored the mechanism of this inhibition. We show that LOX-PP attenuates integrin signaling pathway in Her-2/neu-driven NF639 breast cancer cells. Specifically, ectopic Pro-LOX and LOX-PP expression inhibited fibronectin-stimulated tyrosine phosphorylation of cellular proteins. Importantly, phosphorylation of FAK on Tyr397 and Tyr576, and the activation of its downstream target p130Cas were substantially reduced. Furthermore, these changes were reflected by a reduced amount of endogenous p130Cas in the Triton X-100 insoluble protein fraction and attenuation of fibronectin-activated haptotaxis. Of

note, treatment with recombinant LOX-PP similarly reduced haptotaxis of NF639 and human MDA-MB-231 and Hs578T breast cancer. Thus, the tumor suppressor function of LOX-PP is likely mediated, in part, by blocking FAK/p130Cas-stimulated cell migration. Interestingly, a single nucleotide polymorphism (SNP) in the LOX-PP region with an approximate frequency of 20% has been identified. We are currently investigating whether there is a correlation between this SNP and the risk of developing breast cancer in African American women.

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P65-27: DETECTING SIGNALING PATHWAY ACTIVATIONS THROUGH SINGULAR VALUE DECOMPOSITION-BASED BINARY REGRESSION

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The Ras and TGF-beta (TGF- β) pathways have both been shown to play important roles in mammary gland tumorigenesis. In breast cancers, these two pathways can synergize to promote epithelial-to-mesenchymal transition (EMT) and metastasis. In contrast, in vitro studies in non-transformed mammary epithelial cells have demonstrated that the TGF- β pathway can exert an antagonistic effect on Ras-initiated mammary epithelial cell transformation. The extent to which these two pathways interact during mammary epithelial transformation and hyperplastic progression in vivo remains unclear. We constructed an in vivo interaction model between the Ras and TGF- β pathways using pathway signature-based singular value decomposition (SVD) binary regression. Using a TGF- β pathway microarray signature generated in vitro, we detected TGF- β pathway activation in mouse mammary gland in which oncogenic H-Ras was acutely expressed. Activation of the TGF- β pathway was confirmed by detecting nuclear translocation of Smads in Ras-expressing mammary glands. Similarly, using a Ras pathway signature generated from mammary glands with acute Ras activation, we detected Ras pathway activation in normal mammary epithelial cells treated with TGF- β 1 and TGF- β 3. Consistent with this finding, we demonstrated significantly increased levels of Ras-GTP and rapid MAPK pathway induction in a normal mammary epithelial cell line and in vivo in the mammary gland following TGF- β pathway activation. Our results demonstrate that pathway signatures extracted from in vivo data can accurately identify activation of that pathway both in vivo and in vitro, and vice versa. Using both computational prediction and biochemical validation, we have shown that the TGF- β pathway is activated after acute activation of the Ras pathway in vivo. Further, the Ras-MAPK pathway is turned on after TGF- β pathway activation in murine mammary gland epithelial cells in vitro. In contrast to some prior studies, our results raise the possibility that the TGF- β pathway may synergize with the Ras pathway during Ras-initiated mammary epithelial cell transformation and hyperplastic progression.

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TUMOR SUPPRESSOR GENES II

Poster Session P66

P66-1: TARGETING p53-INDEPENDENT APOPTOSIS IN BASAL-LIKE BREAST CANCERS

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Background and Objectives: The basal-like subset of breast cancers lack expression of estrogen and progesterone receptors and amplification of Her-2. These tumors therefore cannot be treated with effective hormonal and Her-2-directed therapies and consequently are associated with an inferior prognosis relative to other breast cancer subtypes. Basal-like tumors also exhibit frequent mutational inactivation of the p53 tumor suppressor, making them potentially less susceptible to apoptosis induced by therapeutic chemotherapy and radiation. We have recently identified a novel p53-independent cell death pathway in basal-like tumors involving the pro-apoptotic p53 family member, p73. We find that p73 is selectively upregulated in a subset of these cancers, but its activity is repressed through co-expression of the related p53 family member, p63. The goal of this project is to identify a specific and effective means to induce tumor-specific killing of basal-like cancer cells through activation of p73.

Methodology: Given the large body of data demonstrating that p63 and p73 activity are regulated by several kinase/phosphatase pathways, we propose to carry out a comprehensive RNA interference screen to identify endogenous kinases and/or phosphatases whose inhibition will activate p73-dependent apoptosis in basal-like tumor cells.

Results to Date: In this initial award period, we have established a robust luciferase-based assay for high-throughput detection of p73 activation, by developing stable cell lines harboring a series of p73-dependent reporters. The screening approach consists of transfecting small interfering RNAs (siRNAs) directed against kinases/phosphatases, then assaying p73 activation that occurs following target knockdown. Optimization of the assay parameters has been carried out, including transfection efficiency, signal linearity, and signal-to-noise ratio, all of which are critical to successful high-throughput screening. Subsequently, a first round of screening was completed by assaying knockdown of all known human kinases. Several promising candidate kinases have been identified whose inhibition induces p73 activation. These candidates will now be validated in additional screens, followed by functional assays.

Conclusions and Implications: We have identified candidate kinases whose inhibition activates p73 in basal-like cancer cells. In the remaining years of the project, these candidates will undergo validation, and additional candidate kinases and phosphatases will be identified. If validated, these findings suggest that targeted kinase/phosphatase inhibition may represent an effective therapeutic strategy to induce tumor-specific apoptosis of basal-like carcinomas. Inhibition of the kinases/phosphatases identified through this work may thus form the basis for new clinical trials to improve the outcome for women with this refractory form of breast cancer.

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P66-2: INAPPROPRIATE CELL CYCLE PROGRESSION DUE TO THE Cdc25B ONCOGENE TRIGGERS A p53-DEPENDENT CHECKPOINT: A ROLE FOR DNA DAMAGE PATHWAYS IN BREAST ONCOGENESIS

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The Cdc25 family of phosphatases has well-characterized key roles in cell cycle progression. Cdc25B and Cdc25C function in a biochemically similar manner in vitro and both have been implicated in regulating entry into mitosis via removal of inhibitory phosphorylations on the cyclin-dependent kinases, Cdk 1 and Cdk2. A role for Cdc25B, but not Cdc25C, has been suggested in human breast cancer based on three findings. First, there are examples of Cdc25B overexpression in a subset of human breast tumor whereas alterations in Cdc25C levels are not found. Second, Cdc25B, but not Cdc25C, cooperates with activated Ras in cell culture transformations assays. Third, a mouse model in which Cdc25B is targeted for expression in the mammary gland leads to enhanced tumorigenesis.

With this in mind, the role of overexpression of Cdc25B versus Cdc25C in breast tumorigenesis was addressed. Interestingly, ectopic expression of Cdc25B, but not Cdc25C, inhibits cell proliferation in long-term assays. Chimeric proteins generated from the two phosphatases show that the anti-proliferative activity is associated with the C-terminal end of Cdc25B. Indeed, the catalytic domain of Cdc25B is sufficient to suppress cell viability, in a manner partially dependent upon its C-terminal 26 amino acids that is shown to influence substrate binding. Mutation analysis demonstrates that both the phosphatase activity of Cdc25B as well as its ability to interact with its substrates contributes to the inhibition of cell proliferation. These results demonstrate key differences in the biological activities of Cdc25B and Cdc25C due to differential substrate affinity and recognition. In addition, this may provide insight into the oncogenic activity of Cdc25B that is not found with Cdc25C.

Overexpression of Cdc25B can, indeed, be shown to upregulate the activity of Cdk1 and Cdk2, by reducing their level of inhibitory phosphorylation. Consistent with the effects observed in long-term assays, overexpression of Cdc25B can be shown to trig-

ger arrest in multiple phases of the cell cycle, eventually leading to apoptotic cell death. In cells lacking p14ARF, Cdc25B activates p53 and components of the ATM/ATR pathway. The Cdc25B-induced cell cycle arrest in the G1 and G2 phases is both p53- and p21-dependent and can be inhibited by caffeine. Cdc25B also causes the accumulation of cells with an S phase DNA content in a p53-independent manner. Thus, inappropriate cell cycle progression via the Cdc25B oncogene activates both p53-dependent and independent responses that need to be overcome for breast epithelial cells to fully achieve tumorigenic potential. Although the tumor suppressor p53 plays a clear role in the cellular response to DNA damage, the molecular basis for the selective pressure for loss of p53 activity in a subset of breast tumors has remained unclear. These findings provide insight into the multi-step nature of breast cancer development. Further, this provides a molecular explanation for the need for functional loss of p53 during breast tumorigenesis at least in some cases, such as those involving Cdc25B overexpression.

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P66-3: A MICRORNA COMPONENT OF THE p53 TUMOR SUPPRESSOR NETWORK

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A global decrease in microRNA (miRNA) levels is often observed in human cancers, indicating that small RNAs may have an intrinsic function in tumor suppression. To identify miRNA components of tumor suppressor pathways, we compared miRNA expression profiles of wild-type and p53-deficient cells. Here we describe a family of miRNAs, miR-34a-c, whose expression reflected p53 status. Genes encoding miRNAs in the miR-34 family are direct transcriptional targets of p53, whose induction by DNA damage and oncogenic stress depends on p53 both in vitro and in vivo. Ectopic expression of miR-34 induces cell cycle arrest in both primary and tumor-derived cell lines, which is consistent with the observed ability of miR-34 to downregulate a program of genes promoting cell cycle progression. The p53 network suppresses tumor formation through the coordinated activation of multiple transcriptional targets, and miR-34 may act in concert with other effectors to inhibit inappropriate cell proliferation. We are in an effort to further characterize miR-34's role in tumor initiation and maintenance using cancer mouse models.

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P66-4: SUPPRESSION OF BREAST CANCER GROWTH BY RE-ACTIVATION OF ENDOGENOUS MUTANT p53 PROTEIN BY PRIMA-1

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Mutation of p53 tumor suppressor gene is a common event in many different types of tumors, including breast cancers. p53 mutation confers growth advantage to tumor cells since they do not undergo p53-dependent apoptosis and cell-cycle arrest. Inactive mutant p53 (mtp53) protein generally accumulates, providing a potential molecular target that may be reactivated into a conformation capable of arresting tumor growth. PRIMA-1 (p53 reactivation and induction of massive apoptosis) is a recently discovered small molecule that selectively activates mtp53 protein and induces apoptosis in tumor cells (Bykov et al., *Nat Med.* 2002; 8:282-8). Accordingly, we examined whether PRIMA-1 is able to alter mtp53 conformation and induce cell death in T47-D, BT-474, and HCC-1428 breast cancer cells. Immunofluorescent staining with an antibody that recognizes the mtp53 conformation (PAb240) confirmed that all cell lines express mtp53 protein. After PRIMA-1 treatment PAb240 recognition was lost and p53 stained with an antibody that recognized only the wild-type protein PAb246). All cell lines contain estrogen and progesterone receptors. In vitro treatment of tumor cells with PRIMA-1 (0-50 μ M) led to a dose-dependent loss of cell viability in T47-D, BT-474 and HCC-1428 cancer cells. In contrast, PRIMA-1 had no effect on cell viability of MCF-7 cells or normal breast cells that express wild-type (wtp53) protein. In vivo xenograft studies confirmed the in vitro data. We injected tumor cells into nude mice supplemented with a 1.7 mg/60 day release estradiol pellet. PRIMA-1 administration, via tail vein (50 mg/kg) was started when tumor size reached approximately 100 mm³. PRIMA-1 treatment inhibited the growth of T47-D, BT-474, and HCC-1428 xenografts but did not influence xenografts of MCF-7 cells. To determine mechanistically how PRIMA-1 caused tumor regression, we studied its anti-angiogenic properties using human umbilical vein endothelial cells (HUVEC) and found it to be ineffective on cell viability. Since PRIMA-1 brought about a reduction in tumor epithelial cells, which are a rich source of VEGF, we suggest that it may act by suppressing bystander VEGF-secreting epithelial cells, thereby inhibiting angiogenesis and cell survival during tumor regression. We also studied the effects of PRIMA-1 in a DMBA-induced breast tumor model that expresses wild-type p53 protein and found it to be ineffective against these tumors. However, when PRIMA-1 was administered to animals with progestin-accelerated DMBA-induced tumors, almost half of these tumors regressed. Immunohistochemical analysis showed that the majority of progestin-accelerated tumors expressing mutant p53 as determined by using conformation specific antibodies in immunohisto-

chemistry. Thus, our findings in two different models suggest that PRIMA-1 is effective for the treatment of mtp53 expressing breast tumors. Since over 50% of human breast cancer cells contain mtp53 protein, many of which develop resistance to anti-hormone therapy, the use of PRIMA-1 could prove to be an effective strategy for the eradication of mtp53 containing breast cancer cells. We propose therefore that PRIMA-1 should be considered for combination therapy with anti-hormones, aromatase inhibitors, and anti-angiogenesis or vascular disrupting agents.

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P66-5: CRITICAL ROLES OF PHOSPHORYLATION IN ACTIVATING p53 TUMOR SUPPRESSION ACTIVITY

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p53 is required for suppressing the development of various cancers such as breast cancer and plays multiple tumor suppression roles, including cell cycle arrest, apoptosis, cellular differentiation, and senescence. p53 remains in an inert state in normal cells in the absence of stresses, but its activity and stability are greatly induced after various genotoxic and cellular damage. While the mechanism underlying p53 activation remains unclear, accumulating evidence has suggested that posttranslational modifications such as phosphorylation are important to activate p53-dependent tumor suppression. Mouse p53 is phosphorylated at Ser18 and Ser23 after DNA damage. To determine whether these two phosphorylation events have synergistic functions in activating p53 responses, we simultaneously introduced Ser18/23 to Ala mutations into the endogenous p53 locus in mice. While partial defects in apoptosis are observed in p53S18A and p53S23A thymocytes exposed to IR, p53-dependent apoptosis is essentially abolished in p53S18/23A thymocytes, indicating that these two events have critical and synergistic roles in activating p53-dependent apoptosis. In addition, p53S18/23A, but not p53S18A, could completely rescue embryonic lethality of *Xrcc4*^{-/-} mice that is caused by massive p53-dependent neuronal apoptosis. However, certain p53-dependent functions, including G1/S checkpoint and cellular senescence, are partially retained in p53S18/23A cells. While p53S18A mice are not cancer prone, p53S18/23A mice developed a spectrum of malignancies distinct from p53S23A and p53^{-/-} mice. Interestingly, *Xrcc4*^{-/-}p53S18/23A mice fail to develop tumors like the pro-B cell lymphomas uniformly developed in *Xrcc4*^{-/-}p53^{-/-} animals but exhibit developmental defects typical of accelerated ageing. Therefore, Ser18 and Ser23 phosphorylation is important for p53-dependent suppression of tumorigenesis in certain physiological contexts.

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P66-6: TCP80 AND RHA ARE POSITIVE p53 IRES TRANS-ACTING FACTORS POORLY EXPRESSED IN CANCER CELLS WITH DEFECTIVE p53 RESPONSE TO DNA DAMAGE

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The tumor suppressor p53 is essential for the protection of cells against tumorigenic transformation. p53 protein accumulates in the cell following stressful events such as DNA damage. Induction of the p53 protein is known to be regulated at the level of translation. A newly identified internal ribosome entry site (IRES) located at the 5'-UTR of the p53 mRNA controls cap-independent translation of p53 following DNA damage. We have identified two p53 IRES-transactivating factors (ITAFs), TCP80 and RHA, that positively regulate p53 IRES activity in response to DNA damage. Furthermore, we demonstrate that two breast cancer cell lines known to harbor wild-type p53 have defective p53 induction and diminished p53 IRES activity in response to DNA damage. Moreover, the expression levels of TCP80 and RHA are low in these cells, and overexpression of either protein has no effect on p53 IRES activity. A significant increase in p53 IRES activity was observed only when TCP80 and RHA were co-expressed in these two cell lines. Our results suggest that the positive ITAFs of the p53 IRES are important for p53 induction following DNA damage and that defective expression of these ITAFs may be involved in the development of breast cancer.

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P66-7: IDENTIFICATION OF NOVEL TUMOR SUPPRESSOR GENES IN HUMAN BREAST CANCER USING NONSENSE-MEDIATED mRNA DECAY INHIBITION (NMDI)-MICROARRAY ANALYSIS

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Eukaryotes have evolved sophisticated mechanisms to prevent the biosynthesis of mutant proteins that may produce potential deleterious effects and reduce the overall "fitness" of an organism. One such mechanism is nonsense-mediated mRNA decay (NMD), a pathway conserved from yeasts to humans, that targets nonsense mutation bearing transcripts for degradation. Tumor suppressor genes (as well as genes mutated randomly) often harbor nonsense mutations in cancer cells, and the vast majority of the mutant mRNAs are degraded by the NMD pathway.

NMD-microarray analysis is a strategy that can be used to identify truncating (nonsense) mutations in candidate tumor suppressor genes in cancer cells in vitro [1-4]. The purpose of this project was to identify novel genes that harbor nonsense mutations in breast cancer cell lines commonly used as in vitro models in the study of breast cancer biology, with the ultimate aim of identifying novel tumor suppressor genes for sporadic breast cancer. We focused our attention on the long arm chromosome 22, which is known to undergo loss of heterozygosity (LOH) in a significant proportion of sporadic breast tumors. Before the NMD-microarray strategy could be undertaken, we very thoroughly characterized chromosome 22q copy number and allelic imbalance in breast cancer cell lines by integrating publicly available genetic and genomic data with our empirical data obtained by subjecting cell lines to single nucleotide polymorphism (SNP) array analysis using humanhap300-duo Infinium BeadChips [5]. The humanhap300-duo chips contain over 318,000 tag SNPs derived from Phase 1 and Phase 2 HapMap data in a two-sample format. Based on chromosome 22q alterations, MCF-7, T-47D, and MDA-MB-231 breast cancer-derived cell lines were selected for NMD-microarray analysis. Two different regimens for inhibiting NMD were then directly compared for their ability to specifically inhibit the NMD process while having minimal impact upon wild-type transcripts [4]. An improved second-generation NMD protocol was performed [4] and total RNA subjected to gene expression profiling using GeneChip oligonucleotide expression arrays (Affymetrix), the data from which will be presented.

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P66-8: ALTERED FUNCTION p53 MISSENSE MUTATIONS ASSOCIATED WITH BREAST CANCER CAN HAVE SUBTLE EFFECTS ON TRANSACTIVATION

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In human cells the p53 tumor suppressor is a critical, sequence-specific master regulator that directly controls differential induction of target genes through transactivation at promoter response elements (RE). Mutations in p53 that alter transactivation function could result in changes in the strength of gene activation or spectra of genes regulated, thereby leading to dramatic phenotypic changes and diversification of cellular responses.

Mutations in p53 are associated with ~25% of sporadic breast cancers, a rate lower than the frequency in other cancers, such as lung and colorectal carcinomas. However, p53 mutations are more frequent in BRCA1/2 germline-associated familial breast cancers. Notably, when p53 mutations are inherited, especially for clinically defined Li-Fraumeni syndrome (LFS) and Li-Fraumeni-Like (LFL) families, there is a predisposition to early onset cancers, including breast. At the molecular level, cancer-associated p53

mutations are usually associated with defects in cell cycle checkpoints, suppression of transformation by oncogenes, induction of apoptosis and maintenance of genome stability. At the clinical level, p53 mutations in breast cancer are associated with the more aggressive, poor prognosis, hormone receptor-negative subtypes such as the HER2+/ER- and the basal-like subtypes.

The objective of this project is to determine the functionality of p53 mutants found in breast cancers, particularly those associated with BRCA1/2 cancers, LFS/LFL or identified in women undergoing neoadjuvant therapy for locally advanced tumors. An in vivo, isogenic yeast model system that allows for rapid analysis of p53 function in a cellular environment has been utilized to assess the ability of p53 wild type (WT) and mutants to transactivate from 12 REs that are targets for p53 transactivation of human genes. Transactivation capacities of WT and mutant p53 have been determined at variable expression levels using a qualitative and a quantitative reporter to establish a functional fingerprint for each mutant.

Among 50 missense mutants, 31 were classified as loss-of-function mutations. The remaining 19 (38%) showed altered transactivation capacity toward at least one biological RE. Interestingly, at high levels of p53, 8 of the 19 mutants that retain function (L130V, A138V, C141W, R174K, R174W, H214R, V272L, and E285K) look identical to WT in their transactivation capacity. However, when the levels of these p53 mutants were reduced, subtle transactivation defects were revealed.

Our results demonstrate that altered function p53 missense mutations can occur in breast cancers. Although the transcriptional effects associated with these mutations are often subtle, p53 levels can exaggerate such differences. Understanding the consequences of altered function mutations on the p53 transcriptional network will help elucidate how specific mutations contribute to the development, penetrance, and phenotype of breast cancers. We propose that assessments of functional fingerprints for p53 missense mutations associated with breast cancer are relevant to the tailoring of individual therapies, especially when the treatment agents impact p53-dependent biological responses.

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P66-9: A MICRORNA COMPONENT OF THE p53 TUMOR SUPPRESSOR NETWORK

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A global decrease in microRNA (miRNA) levels is often observed in human cancers, indicating that small RNAs may have an intrinsic function in tumor suppression. To identify miRNA components of tumor suppressor pathways, we compared miRNA expression profiles of wild-type and p53-deficient cells. Here we describe a family of miRNAs, miR-34a-c, whose expression reflected p53 status. Genes encoding miRNAs in the miR-34 family are direct transcriptional targets of p53, whose induction by DNA damage and oncogenic stress depends on p53 both in vitro and in vivo. Ectopic expression of miR-34 induces cell cycle arrest in both primary and tumor-derived cell lines, which is consistent with the observed ability of miR-34 to downregulate a program of genes promoting cell cycle progression. The p53 network suppresses tumor formation through the coordinated activation of multiple transcriptional targets, and miR-34 may act in concert with other effectors to inhibit inappropriate cell proliferation. We are in an effort to further characterize miR-34's role in tumor initiation and maintenance using cancer mouse models.

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P66-10: PILOT STUDY ON FACTORS SECRETED BY DIFFERENTIATING MAMMARY EPITHELIAL CELLS (MECS) THAT CAN SUPPRESS PROLIFERATION OF BREAST CANCER CELLS

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In normal cells, proliferation and differentiation are regulated and coordinated in a way that activation of differentiation in normal cells is typically associated with cessation of proliferation. However, the balance between the two is usually disrupted in tumorigenesis, ultimately leading to a complex cellular phenotype typical of cancer cells. Our lab previously demonstrated that BRCA1-depleted mammary epithelial cells (MECs) failed to form acinus structure, i.e., differentiate in 3D Matrigel culture system. We further showed that formation of acinus structure can be rescued by culturing the cells with conditioned medium collected from differentiating MECs, suggesting certain factor(s) secreted by MECs might serve in a paracrine/autocrine fashion to suppress cell proliferation but induce cell differentiation. In attempts to identify the differentiation-inducing factors in conditioned medium, conditioned medium was fractionated and applied to breast cancer cell, MCF7, cultured in Matrigel. Unexpectedly, a cytotoxic activity was observed on MCF7 cells treated with the fraction of molecular weight

between 10-50kDa after 7 days. In the past year, I first confirmed the cytotoxicity on various breast cancer cell lines with different genetic backgrounds, including MD-AMB-468 and SKBR3. From mass spectrometric analysis on this 10-50kDa fraction of the conditioned medium, six factors such as interleukins (ILs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and other cytokines, which are known to be involved in cell proliferation and death, have been selected as proteins of interests and their cytotoxic activities were confirmed by immunodepletion. HEK293T cells overexpressing each of the six factors have been established for future reconstitution experiments and animal studies.

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P66-11: THE ROLE OF THE Wwox TUMOR SUPPRESSOR IN BREAST CANCER

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The expression of the Wwox tumor suppressor protein is highly reduced or lost in breast cancer cells and tissues, in part due to hypermethylation in the WWOX regulatory region. In this study, we have further investigated: (1) The effect of Wwox restoration through either overexpression of the exogenous cDNA, or treatment of DNA methyltransferase inhibitor, on growth and apoptosis and (2) the Wwox signal pathways in regulation of tamoxifen-resistance in breast cancer development. We found that Wwox restoration suppressed growth of Wwox-deficient breast cancer-derived cells through activation of the intrinsic caspase pathway and Wwox restoration by intratumoral administration of either Ad-WWOX or 5-aza-2'-deoxycytidine, suppressed tumor growth in nude mice by inducing apoptosis. Lost or reduced expression of Wwox and progesterone receptor (PR) and high level of expression of Prkaria, Ap2γ, and ErbB2 was significantly correlated with tamoxifen resistance. Reduced Wwox expression was better than PR in prediction of resistance, especially in high-risk patients, and nuclear Ap2γ expression was better than ErbB2, especially in low-risk patients. A study with a larger cohort of about 800 breast cancer cases to examine the same markers is under way to validate the above findings.

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P66-12: FoxP3 IS AN X-LINK TUMOR SUPPRESSOR GENE IN BREAST CANCER

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FoxP3 is among the newest members of the forkhead winged helix family. It was identified during position cloning of Scurfin, a gene responsible for X-linked autoimmune diseases in mice and humans (immune dysregulation, polyendopathy, enteropathy, X-linked). The mutation in mice and those in some human IPEX patients are analogous as they cause frameshift and early termination of translation. Further studies indicated that the gene is responsible for the development of Treg, which explains, at least in part, the autoimmune phenotype of diseases.

In our analysis of the immune functions of mice heterozygous for the FoxP3 mutation, we observed a high rate of spontaneous mammary cancer. The heterozygous female mice are also substantially more susceptible to carcinogen DMBA. These data indicated that Foxp3 may play an important role as a tumor suppressor gene in breast cancer.

Meanwhile, a recent study indicated that mice with a targeted mutation of NFAT4 also developed spontaneous mammary cancers. Since NFAT is an essential partner of FoxP3 in immune regulation, it is of great interest to determine whether a similar interaction is responsible for the observed tumor suppressive activity of FoxP3. So far, the role that FoxP3 and NFAT may play in breast cancer development has not been reported. NFAT family members may be the key partners in the tumor suppressive function of FoxP3 in breast cancer development. Therefore, deciphering the mechanism of how FoxP3 and NFAT function in tumor formation will help understand the cause of the pathogenesis and prevent or treat breast cancer.

In our study, we found the FoxP3 gene was expressed in breast epithelial cells but downregulated in mammary cancer tissues. In the meantime, overexpression of FoxP3 in a variety of breast cancer cells resulted in a substantial inhibition of their growth. Furthermore, FoxP3 inhibited the transcription of ErbB2, the major oncogene for breast cancer, by targeting and repressing the ErbB2 promoter. Our further analysis demonstrated that the growth inhibition was completely reversed by constitutive expression of the ErbB2 gene. The significance of the genetic defects in human breast cancer has been demonstrated in multiple lines of evidence, including: deletion (12.8% of 232 samples tested), somatic mutation (35% of 65 cases sequenced), and lack of expression (80% of more than 600 cases tested). Our data revealed that FoxP3 is an important breast cancer suppressor gene in mice and humans.

Our analysis of NFAT4 function also provided evidence supporting its role as a tumor suppressor. Our data showed that NFAT4 repressed Erb2 transcription by measuring ErbB2 reporter activity, suggesting NFAT4 may serve as a repressor for ErbB2/Her-2 promoter. NFAT4 was found in a complex with FoxP3, indicating they may functionally play important roles together. Our further analysis also found a decrease of NFAT4 expression in some mice breast cancer samples. In summary, our data suggest that FoxP3 and NFAT4 are important breast cancer suppressor genes in the mouse.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0532.

P66-13: CELLULAR RETINOL BINDING PROTEIN-1 (CRBP1) IS A GROWTH REGULATOR IMPORTANT FOR PARITY-MEDIATED PROTECTION IN THE MAMMARY GLAND

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Despite current advances in the treatment of breast cancer, it is still the most common type of cancer death seen in women and will likely require advances in prevention to reduce this statistic. Of interest, women who give birth to a child before 20 years of age demonstrate a decrease in their overall lifetime risk of developing breast cancer. A similar phenomenon is observed in rodents. The mechanism by which this protection occurs is still largely unknown although increases in p53 responsiveness and decreases in estrogen receptor-positive cell proliferation, likely through transforming growth factor beta (TGF β) activity, have been noted. In a screen for genes that are upregulated in response to estrogen and progesterone, we identified the cellular retinol binding protein-1 (CRBP1) as a gene that is increased with similar kinetics to the responsiveness of p53. CRBP1 is a gene that often hypermethylated during breast cancer progression and is underexpressed in 24% of human breast carcinomas. The literature suggests that this protein may have a dual role as a protein involved in retinoic acid metabolism and as a tumor suppressor. Our studies were designed to examine the role of CRBP1 in mediating the protection of the mammary gland and mammary epithelial cells in human and rodent systems.

Our experiments demonstrate that CRBP-1 is persistently upregulated in the mammary gland in response to parity in both human and rodent models. Loss of CRBP-1 did not appear to have a significant impact on p53 signaling except the p53 activity induced through TGF β treatment. TGF β -induced signaling to caga-luc or 3TP-luc reporter constructs was significantly impaired by the reduction of CRBP1. An interesting feedback network was observed between CRBP1 and insulin-like growth factor-1 (IGF-1), which is a growth factor known to increase one's susceptibility to breast cancer. IGF-1 treatment blocked the upregulation of CRBP1 by pregnancy levels of estrogen and progesterone in mammary gland organ cultures. Furthermore, a knockdown of CRBP1 in human mammary epithelial cells led to an increased proliferation in response to IGF-1.

Our data suggest that CRBP1 expression may play a role in parity-induced protection through regulation of proliferative signals, either through facilitating TGF β signaling or abrogating IGF-1-induced responses. Taken together with its loss during the progression to breast cancer, we believe that CRBP1 may be of significant importance for protection within the mammary gland.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0634 and Rays of Hope.

P66-14: EXAMINING THE ROLE OF DOWN SYNDROME CRITICAL REGION 1 GENE IN MAMMARY CELL PROLIFERATION

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Breast cancer is the second leading cause of cancer death among women. Intriguingly, women with Down syndrome exhibit a dramatically reduced occurrence in breast cancer compared to age-matched controls (Satge et al., 1998; Hasle et al., 2000; Satge et al., 2003) with protective effects observed at all age groups. Breast cancer is rarely observed in Down syndrome women even though the average life expectancy of these patients is now over 50 years of age. Down syndrome patients also exhibit reduced incidence of solid epithelial tumors in general although none as strikingly affected as breast cancer. These observations suggest that a gene or genes present on chromosome 21 can function as a tumor suppressor when overexpressed. A potential candidate for a chromosome 21-encoded antitumorigenic protein is DSCR1 (Down syndrome critical region-1). DSCR1 is highly expressed in mammary tissue (Ernak et al., 2001) and has been shown to regulate the calcium-dependent protein phosphatase calcineurin. Calcineurin has been shown to play a critical role in promoting the proliferation of numerous cell types. The goal of this research is to test the hypothesis that DSCR1, via its ability to modulate calcineurin function, can regulate mammalian breast cancer cell proliferation. We have found that inhibition of calcineurin with CsA reduces MCF7 cell proliferation as measured by MTT assays or crystal violet staining. We are currently assaying the proliferation of DSCR1-overexpressing MCF7 cells. Since loss of

DSCR1 may lead to either enhanced calcineurin function or loss of calcineurin function, we have also investigated the consequences of inhibiting DSCR1 expression. Although we detect a slight elevation of cyclin D expression in DSCR1-depleted MCF7 cells, disruption of DSCR1 expression with shRNA did not significantly alter MCF7 cell proliferation. These results indicate that DSCR1 is not essential for MCF7 cell proliferation. Experiments testing the effects of DSCR1 overexpression on MCF7 cell proliferation are under way to determine if DSCR1 contributes to the protective effects of Down syndrome for breast cancer development.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0594.

P66-15: ABSTRACT WITHDRAWN

P66-16: DETECTION OF TUMOR SUPPRESSOR GENE MUTATIONS IN DCIS USING OLIGONUCLEOTIDE RESEQUENCING ARRAYS

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Background and Objectives: The molecular events that trigger DCIS to progress to IDC are not understood; however, LOH at 17p is a recurrent observation specific to grade III DCIS and IDC suggesting a role for these alterations in tumor progression. High-density oligonucleotide arrays offer the ability to sequence large numbers of loci in parallel using an automated approach. Our plan is to use this technology to search for mutations at 17p13 in IDC cells that display loss of this region. To do this we: (1) identified IDCs with 17p13 loss using SNP-CGH, (2) designed and validated primers to amplify all 1389 exons that map to 17p13, (3) designed a resequencing array to detect mutations and (4) validated mutations found in IDCs in a cohort of DCIS.

Results to Date: We have identified IDCs with 17p13 loss using SNP-CGH (Figure 1). Unique sequence primers have been designed and validated to amplify all coding sequences on 17p13. An array has been designed to sequence all coding sequences on 17p13. The specificity of the primers for the arrayed exons (Figure 2). Software has been developed for automated design of primers to eliminate all repetitive and homologous sequences. Software to eliminate known SNPs and detect protein modifying alterations has been developed.

Conclusions: The consequences of this analysis will demonstrate the effectiveness of DNA sequencing arrays in the identification of TSGs in breast cancer. This will open up new possibilities for following the same procedure in other regions showing frequent LOH and so identifying breast cancer-associated genes.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0641.

P66-17: IDENTIFYING CANDIDATE BREAST TUMOR SUPPRESSORS USING INHIBITION OF NONSENSE MEDIATED MRNA DECAY IN MCF-10A CELLS TRANSFORMED BY RANDOM MUTAGENESIS WITH FRAMESHIFT MUTAGEN ICR191 AND SELECTING IN TISSUE CULTURE CONDITION THAT MIMIC TUMOR ENVIRONMENT

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To develop an in vitro model for analysis of genetic alterations associated with breast carcinogenesis we used random mutagenesis and selection of human nontumorigenic immortalized breast epithelial cells MCF-10A in tissue culture conditions that mimic tumor environment. Random mutations in MCF-10A cells were generated with frameshift-inducing agent ICR191. Selection of transformed cells was performed in

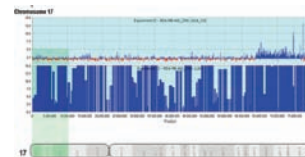


Figure 1: Copy number and LOH data from the breast cancer cell line MCF-10A using the SNP-CGH array. All of chromosome 17 is demonstrated. LOH at 17p13 is highlighted in green. It can be seen that this region is showing copy number loss (red) as well as LOH (blue) graph. This is in contrast to a homologous region of the G arm, where LOH is increased, with an accompanying amplification. This data was graphed using a computer Copy Number Tool (Copy-NumberTool).

Figure 1: SNP-CGH showing LOH on 17p13.

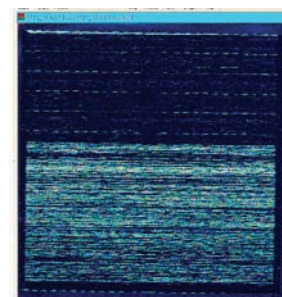


Figure 2: Custom Array for Chromosome 17p Demonstrating Specific Hybridization: The hybridization cocktail contained "spike in" PCR products that would bind to sequences covering roughly half of the array. The bands visible in the upper half of the array are 62 grid alignment sequences. The illuminated band across the top of the array is a positive control plasmid sequence (GEX) that is spiked into the hybridization cocktail. This experiment demonstrates the specificity of the PCR products for the sequences tiled on the array.

Figure 2: Hybridization specificity of resequencing array.

two steps. The first selective step was cell cultivation for 6 months in tissue culture medium containing mutagen at concentration that induced in the MCF-10A cells a DNA damage signaling causing in mutagenized cells a p53 protein accumulation but still allowed cell replication. The second step of selection was either cell cultivation in the medium with reduced growth factor supply or cell cultivation in the medium, which mimics hypoxia condition or anchorage-independent growing in soft agar. Using this strategy of mutagenesis and selection, we have generated several independently derived cultures with various degrees of tumorigenicity. Gene Identification by Nonsense-mediated mRNA decay Inhibition (GINI) analysis has identified TP53, smoothelin, RASSF6, and other genes containing ICR191-induced frameshift mutations in the transformed MCF-10A cells. The p53 gene mutations resulting in the loss of protein expression had been found in all of the independently transformed MCF10A cultures, which can form large progressively growing tumors with sustained angiogenesis in nude mice. Identifying genes containing bi-allelic ICR191-induced frameshift mutations in the transformed MCF-10A cells generated by random mutagenesis and selection indicates putative breast tumor suppressors and provides a model for studying the role of mutant genes in breast carcinogenesis.

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P66-18: IDENTIFICATION OF A CELL SENEESCENCE GENE SEN16 AT 16Q24.3 FOR BREAST TUMOR CELLS

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Breast cancer cells can proliferate indefinitely, whereas normal diploid breast epithelial cells undergo replicative senescence after a finite number of generations. Cellular senescence is regulated through the functioning of multiple genes and escape from senescence is hypothesized as an essential early step in tumor progression. Molecular pathways leading to cell senescence have been contemplated as potential targets for cancer therapy.

Numerous cytogenetic and molecular studies of breast and other cancers revealed frequent loss of heterozygosity on the long arm of chromosome 16, suggesting the presence of tumor suppressor gene(s). Applying a deletion strategy, following the microcell mediated transfer of a normal intact chromosome 16 into immortal breast tumor cells; we progressively narrowed the position of a cell senescence gene, SEN16, at 16q24.3. Precise positional information led to the identification of YAC and BAC clones for SEN16 locus. Introduction of YAC and BAC clones into breast tumor cells allowed us to identify a BAC clone that restored normal cell growth pattern and senescence in tumor cells. Human genome database search for the transcripts encoded from SEN16 locus identified five candidate cDNAs for cell senescence/tumor suppressor genes. The sequence information of candidate cDNAs was used to isolate full-length cDNA clones, encoded from SEN16 locus. While multiple transcript variants are encoded for candidate cDNAs in normal and tumor cells, the variants expressed in immortal tumor cells were different from those in normal cells. It is interesting that each of these cDNAs is encoded as part of a single transcript, as well as independent open reading frames. The expression of each of the transcripts was verified by RT-PCR in normal epithelial cells. Individual cDNAs representing different open reading frames were cloned in a mammalian cell expression vector, in frame with FLAG tag, and tested for the restoration of senescence. Ectopic expression of one of the cDNAs (designated AIMC1) in breast tumor cell lines led to terminal growth arrest and senescence. Analysis of breast and other tumor cell lines revealed genomic rearrangements at SEN16 locus, as well as loss of expression of the complementing cDNA. These results suggest that the cDNA that we have cloned indeed represents a tumor suppressor gene. In silico analysis of the cDNA sequence revealed that predicted protein is a part of an ubiquitin-ligase complex that carries F-box motifs. Proteins containing F-box motifs function in protein degradative pathways and are known to interact with cell cycle proteins. Malfunctioning of these pathways may contribute to the deregulation of cell cycle, leading to the development of cancer. Further characterization of SEN16 gene for the identification of signaling pathways will afford insight into deregulation of cell growth and senescence.

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P66-19: XANTHINE OXIDOREDUCTASE IS DOWNREGULATED IN INVASIVE MAMMARY EPITHELIAL CELLS

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Introduction: Xanthine oxidoreductase (XOR) may exert an important, but poorly defined, role in the pathogenesis of breast cancer (BC). Loss of XOR expression has been linked to aggressive hepatic, renal, gastric, and mammary cancer. In both mouse models of mammary carcinogenesis and in human BC patients, decreasing mammary epithelial cell (MEC) XOR was associated with and/or predictive of poor clinical out-

come and the degree of BC aggressiveness. Patients without evident epithelial XOR expression had the most aggressive BC and had 2.5-fold increased risk of recurrence compared with patients expressing normal or modestly reduced XOR. A similar decline in serum XOR has been linked to aggressive stage in BC patients.

Description and Hypothesis: These data suggested the possibility that down-regulation of XOR may be functionally linked to aggressive BC. The goal of the present investigation was to determine whether the down-regulation of XOR observed in clinically aggressive BC was an intrinsic property of highly invasive MEC. We studied the role of XOR expression on migration/motility in vitro using HC11 normal mouse MEC, HB4a normal human MEC, and the human mammary tumor cell lines MCF-7 and MDA-MB-231.

Summary of Current Results: Data shown here demonstrate vigorous XOR expression in normal or weakly invasive MEC that is lost in highly invasive MEC cells. Furthermore, overexpression of recombinant XOR cDNA in highly invasive MEC inhibits their migration/motility in vitro, whereas pharmacological inhibition of XOR in MEC possessing high XOR expression induces their migration/motility in vitro.

Conclusion: These data reveal a functional link between XOR expression and the migration/motility of MEC, and they suggest a potential link between expression of XOR and suppression or inhibition of mammary tumorigenesis and/or metastasis.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9149 and The Robert and Helen Kleberg Foundation.

P66-20: MASPIN ACTS ON CELL SURFACE AND IN THE CYTOPLASM TO SUPPRESS TUMORIGENESIS AND TUMOR METASTASIS

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Maspin is a tumor suppressor serpin (serine protease inhibitor) that inhibits tumorigenesis, tumor invasion, and migration. We discovered that maspin at different cellular locations could act to control cell apoptosis and cell-matrix adhesion. We found that mammary tumor cells transfected with maspin undergoes apoptosis through the mitochondrial death pathway. Translocation of maspin to the inner membrane of mitochondria is linked to the opening of the PT (permeability transition) that in turn causes the loss of trans-membrane potential (Ψ_m) thus commencing apoptotic degradation. Suppression of maspin overexpression by RNA interference desensitizes cells to apoptosis. On the other hand, extracellular maspin increased MCF-10A cell adhesion to the matrix, and conversely both an anti-maspin antibody and maspin knockdown by RNA interference resulted in decreased cell adhesion. Mutation analyses revealed that a region of 86 amino acids located between aa139 and aa225 was responsible for maspin effect on adhesion. Function-blocking antibody against $\beta 1$ prevented maspin-dependent increase in cell adhesion, and co-immunoprecipitation assay demonstrated the association of maspin with $\beta 1$ integrin, indicating that maspin is functionally and physically associated with $\beta 1$ integrin. These studies uncover the molecular mechanism of tumor suppression by maspin.

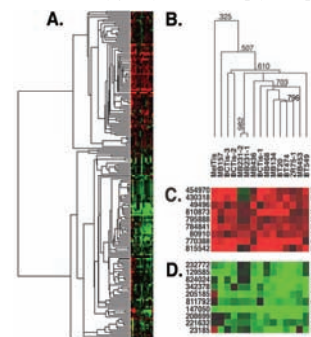
This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8029.

P66-21: NO-DETECTABLE AND DECREASED EXPRESSION OF CHROMOSOME 6-ENCODED PHOSTENSIN TRANSCRIPTS IN HUMAN BREAST CANCER

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Objective: Cytogenetic aberration and loss of heterozygosity (LOH) imply the presence of tumor suppressor genes encoded by human chromosome 6 DNA. Functional suppression of tumorigenicity by introduction of human chromosome 6 into breast cancer cell lines demonstrates that chromosome 6-encoded tumor suppressor genes have yet to be identified. MDA-MB-231 is a human metastatic breast cancer cell line and MDA/H6 is a MDA-MB-231 derivative cell line that contains a neo-tagged human chromosome 6 and displays no tumorigenic phenotype in athymic nude mice.

Methods: cDNA microarrays of 30,000 cDNAs with 19,592 unique genes were used to compare expression profile of MDA/H6 with breast



Microarray identification of EST208699 downregulated in 10 breast cancer cell lines and 3 tumor tissue specimens

cancer cell lines (n=10) and tumor tissues (n=3). Quantitative RT-PCR was used to verify microarray-detected expression changes. Northern blots were employed to detect alterations in transcript sizes and expression levels.

Results: Out of 19,592 genes tested, 180 were differentially expressed in breast cancer cell lines and tumor tissue specimens. Four differentially expressed genes are known to encode by human chromosome 6 DNA. Particularly, beta-tubulin (TUBB) and an expression sequence tag (EST208699) at 6p21 and high mobility group nucleosomal binding domain 3 (HMGN3) at 6q14.3 were significantly downregulated in MDA-MB-231, in contrast to the chromosome 6-mediated suppressed cell line MDA/H6. Of particular interest, expression of EST208699 in 10 breast cancer cell lines was 5-fold lower than in MDA/H6 confirmed by qRT-PCR analysis. Northern blots demonstrated that this novel gene encodes 4 transcripts. In contrast to MDA/H6 cell line, the largest transcript (4-Kb band) was absent in all 10 malignant breast cancer cell lines and all smaller bands showed decreased expression. Blast search aligned this EST with protein sequence KIAA1949 identical to phostensin encoded by 6p21.3, a band region with frequent alterations in breast cancer cells. Phostensin is a regulatory subunit that targets protein phosphatase-1 to F-actin cytoskeleton.

Conclusion: Our study revealed multiple chromosome 6-encoded genes that were down-regulated in malignant breast cancer cell lines. The demonstration of abnormal expression of the 6p21.3-encoded phostensin gene in 10 breast cancer cell lines in contrast to no tumorigenic cell line MDA/H6 implies a tumor suppression activity that deserves further study.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-97-1-7236; National Institutes of Health (NIDDK-06-925); and Catherine McCormick Genomics Center.

P66-22: SYSTEMATIC EXPLORATION OF CELL ADHESION AND CYTOSKELETAL GENES AS TUMOR SUPPRESSORS IN BREAST CANCER

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Background and Objectives: Given the known examples of adhesion/cytoskeletal gene inactivation in breast cancer (e.g., E-Cadherin), we reason that there exist additional cell adhesion and cytoskeletal genes that act as tumor suppressor genes (TSGs) in breast cancer. The objective is to identify novel cell adhesion and cytoskeletal TSGs in breast cancer.

Brief Description of Methodologies: (1) Identification of all adhesion and cytoskeletal genes in human genome – By analyzing the Gene Ontology (GO) database, we identified all the adhesion and cytoskeletal genes in the human genome as the starting point for discovery. (2) Four genomics-based “filters” to prioritize adhesion and cytoskeletal genes for TSG candidacy – (i) DNA copy number loss – for systematically searching for all the known adhesion and cytoskeletal genes coinciding with DNA copy number loss using array comparative genomic hybridization (CGH) data collected from primary human breast tumors (Hicks et al. *Genome Res.* 16:1465 (2007)). (ii) Correlation of DNA copy number and RNA expression – Pearson correlation coefficients were calculated for all genes in a panel of 33 human breast cancer cell lines using array-based measurements of DNA copy number and RNA expression. A positive correlation coefficient indicates DNA copy number-dependent RNA expression, and a candidate TSG with a positive correlation coefficient would be assigned higher priority. (iii) Status of somatic point mutations per the study of Wood et al. (*Science* 318:1108 [2007])—Wood et al. sequenced the coding exons of all 18,191 Reference Sequence (RefSeq) genes in 11 human breast tumors. We pose that somatic point mutations identified by Wood et al. may be used as corroborating evidence to support a candidate TSG that coincides with a DNA copy number loss. (iv) Gene methylation status – since methylation is a common “2nd hit” to inactivate a TSG in cancers, we reason that genome-wide gene methylation survey of breast cancer (Kamalakaran et al. submitted) may be queried as a final genomics-based filter in prioritizing candidate TSGs.

Results to Date: (1) In the GO database, we have identified 1,609 genes related to cell adhesion and cytoskeleton (635 adhesion; 974 cytoskeleton). (2) With the 635 adhesion genes, we did not identify any genes that fit all the four criteria: recurrently deleted in two or more breast tumors, positive correlation of DNA copy number and RNA expression, persistently methylated in more than 50% of breast tumors surveyed (and unmethylated in more than 80% normal breast tissues studied), and harboring somatic point mutations. However, out of 974 cytoskeletal genes, gelsolin was the sole gene that met all four criteria. Gelsolin is a critical regulator of the actin cytoskeleton dynamics and was proposed to be a candidate TSG based on widespread loss of expression in breast cancer (Asch et al. *Cancer Res.* 56:4841 [1996]). Our study substantiates the TSG candidacy of gelsolin by linking it with two forms of primary genetic alterations (gene deletion and point mutation).

Conclusions: As far as we know, this study represents the first systematic exploration of all adhesion and cytoskeletal genes for potential TSGs in breast cancer. This approach is, in principle, transplantable and could be used to discover novel and crucial players of different classes of genes in the development and evolution of mammary neoplasia.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0495.

BRCA1 AND BRCA2 TUMOR SUPPRESSORS II

Poster Session P67

P67-1: THE ROLE OF BRCA1 PROMOTER METHYLATION IN DETERMINING CHEMOSENSITIVITY IN VITRO

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Background: Several groups have demonstrated that women with *BRCA1* germline mutations are more likely to have breast cancers that are basal-like by gene expression profiling. While *BRCA1* germline mutations are uncommon and contribute to fewer than 5% of breast cancer cases, epigenetic alterations in *BRCA1* occur with much greater frequency. Our lab has previously demonstrated that methylation of the *BRCA1* promoter occurs in almost 50% of high-grade, hormone receptor negative sporadic tumors. Given the role of *BRCA1* in both DNA repair and cell cycle regulation, it is likely that cells deficient in *BRCA1* secondary to methylation will be sensitive to DNA damaging agents and resistant to microtubule inhibitors, as has previously been shown for cells deficient in *BRCA1* secondary to mutation. The role of *BRCA1* methylation in determining chemosensitivity is unknown.

Methods: Using an in vitro model, the relative sensitivity of *BRCA1* methylated, mutated, and competent cells was determined using four representative breast cancer cell lines: UACC-3199 (methylated *BRCA1*), HCC-1937 (mutated *BRCA1*), MCF-7 (wildtype *BRCA1*, ER positive) and MDA-MB-231 (wildtype *BRCA1*, ER negative). Exponentially growing cells were treated with cisplatin (CDDP) and paclitaxel. Cells were harvested 96 hours after drug exposure and stained with Annexin-V and DAPI. Cell survival was determined by flow cytometry using FACS DiVa. FlowJo FACS analysis software was used to generate percent apoptotic and live cells. IC50 values and 95% confidence intervals were calculated from dose response curves.

Results: The IC50 values and 95% confidence intervals for CDDP for the UACC-3199, HCC-1937 and MDA-MB-231 cells were 7.4 μ M (4.94-10.9), 14.1 μ M (11.6-16.4), and 21.8 μ M (18.2-30.0), respectively. The IC50 value for CDDP for MCF-7 was not reached, even at a dose of 250 μ M. The IC50 values and 95% confidence intervals for paclitaxel for the UACC-3199, HCC-1937, and MDA-MB-231 cells were 1.8 μ M (1.1-3.8), 2.5 μ M (1.1-4.9) and 0.13 μ M (0.09-0.16), respectively. Despite dose escalation to a paclitaxel concentration of 200 μ M, the IC50 value for MCF-7 was not reached.

Discussion: Previous studies have demonstrated that cells deficient in *BRCA1* secondary to mutation are sensitive to cisplatin and resistant to paclitaxel, as compared to *BRCA1* competent cells. We have demonstrated for the first time that cells deficient in *BRCA1* secondary to promoter methylation are also highly sensitive to cisplatin and resistant to paclitaxel. As almost 50% of high-grade hormone receptor negative tumors have *BRCA1* promoter methylation, *BRCA1* methylation may represent both a novel therapeutic target for platinum-based chemotherapy and a mechanism for acquired resistance to paclitaxel chemotherapy.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0545.

P67-2: FUNCTIONAL ASSAYS FOR CLASSIFICATION OF BRCA2 VARIANTS OF UNCERTAIN SIGNIFICANCE

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¹Mayo Clinic and Foundation, Rochester, ²University of Utah, and ³International Agency for Research on Cancer

Assessment of the influence of many rare BRCA2 missense mutations on cancer risk has proved difficult. A multifactorial likelihood model that predicts the odds of cancer causality for missense variants is effective, but is limited by the availability of family data. Thus, when a missense mutation is identified in the BRCA2 breast and ovarian cancer predisposition gene during clinical testing, the patient with the mutation and the patient's family members cannot benefit from improved risk assessment and counseling with regard to prognosis and medical and surgical prevention and therapeutic measures. The difficulty in interpreting whether missense mutations predispose to cancer stems from the undefined influence of these mutations on BRCA1 and BRCA2 function, in contrast to truncating mutations, which are unconditionally deleterious. The extent of the problem is evident when considering that over 1,100 unique BRCA2 missense mutations have already been identified in over 6,000 families in the USA alone.

As an alternative we developed functional assays that measure the influence of missense mutations on the ability of BRCA2 to repair DNA damage by homologous recombination and to control centriole amplification. We evaluated 22 missense mutations from the BRCA2 DNA binding domain (DBD) that were identified by clinical genetic testing in multiple breast cancer families using these assays and compared the results to those from the likelihood model. Mutations were inserted into a full-length FLAG tagged BRCA2 expression construct and the wild-type and mutant genes were transiently expressed in VC-8 BRCA2 deficient cells. Cells were evaluated for homologous recombination DNA repair activity using a GFP reporter assay. In parallel, the influence of the mutations on centrosome amplification, a measure of cell cycle integrity, was evaluated in HEK 293 cells.

Thirteen variants inactivated BRCA2 function in at least one assay and seven had no effect on BRCA2 function. Of 10 variants with odds in favor of causality in the likelihood model of 50:1 or more and a posterior probability of pathogenicity of 0.99, eight inactivated BRCA2 function and the other two caused splicing defects. Four variants and four controls displaying odds in favor of neutrality of 50:1 and posterior probabilities of pathogenicity of at least 1×10^{-3} had no effect on function in either assay. The strong correlation between the functional assays and likelihood model data suggests that these functional assays are an excellent method for identifying inactivating missense mutations in the BRCA2 DBD. The assays may be a useful addition to models that predict the likelihood of cancer in carriers of missense mutations. This information may prove useful for carriers of these mutations in terms of risk assessment for cancer and selection of preventative and therapeutic options. Efforts to expand the collection of families and family members for co-segregation studies are continuing as are functional studies of a large number of missense mutations.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0480.

P67-3: STUDYING MAMMARY TUMOR METASTASIS USING BRCA1 CONDITIONAL MUTANT MICE

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Background: Cancer metastasis is a fatal problem that causes most cancer mortality. However, the mechanisms underlying tumor metastasis remain illusive. There are several models, but none is well established. To address this, we have used a mouse model that carries a mammary tissue-specific mutation of the breast cancer associated gene-1 (*BRCA1*), and nude mice to study the metastasis of breast cancer.

Materials and Methods: (1) The Brca1Co/Co; MMTV-Cre;p53 mice were divided into two groups, with mammary tumor surgically removed at palpable stages (0.3-0.5 cm in diameter) in one group and no removal of the original tumors in the control group. The animals were sacrificed when tumors reach 2 cm, or animals become sick. (2) Primary mammary tumor cells were implanted into nude mice. Tumors were removed and after 1.5-2 months the nude mice were sacrificed. Microarray analyses were done for gene expression.

Results: (1) In 25 Brca1Co/Co; MMTV-Cre;p53 mice, whose primary tumors were surgically removed at palpable stages, 13 mice demonstrated tumor metastasis. However, in the control group, only 15/47 (32%) mice showed tumor metastasis. In both cases, lung, liver, and spleen are the major organs for tumor metastasis. There is also an increase in tumor burden from 3.4/mouse (non-surgery) to 5.2/mouse (surgery). In the nude mice group, we observed tumor metastasis in 25/38 (65.8%) mice after 1-2 months of surgery, among them most were metastasized to lung (22/25). (2) We have found significant change of gene expression between palpable mammary tumors, whose removal caused metastasis and those, whose removal did not cause metastasis, and between metastasized liver or lung tumor and primary tumor, respectively, as well as between large (>2 cm) and small mammary tumors. (3) Data validation: We have confirmed some gene changes from microarray analysis, e.g., decreased expression of KiL, increased expression of Sypl, Tmod3 and Feh in metastatic tumors compared with non-metastatic tumors; increased expression of Alcam and Icam, and decreased expression of Igfbp4 in the metastatic lung tumors compared to their original mammary tumors.

Conclusion: Our data indicated that surgical removal of primary tumors plays a role in promoting tumor metastasis. We also revealed gene expression changes in tumors of metastatic potential versus that of non-metastatic potential, and metastatic lung tumors and their original mammary tumors. These observations suggest that tumor metastasis may be associated with multiple events.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0597 and National Institutes of Health.

P67-4: GENETIC SCREENING FOR NOVEL FACTORS INVOLVED IN DNA DAMAGE RESPONSE PATHWAY MEDIATED BY TUMOR SUPPRESSOR BRCA1

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Salk Institute

BRCA1 and BRCA2, were identified as the hereditary breast and ovarian cancer susceptibility genes that can account for almost all the entirety of inherited cases of breast cancers. To study the biological function of the tumor suppressor BRCA1, we have proposed to perform genetic screening using a lentiviral vector-based cDNA library that expresses 17,500 full-length human and mouse genes. The library features a number of novelties over traditional ones, including broader target cell range, high-efficiency,

high-quality, easier recovery of target genes and full-length cDNAs. This methodology will not only uncover genes involved in BRCA1 tumor suppressor function but also lead to a general application to functional studies in cancer biology.

BRCA1-deficient HCC1937 cells are unusually sensitive to DNA damage induced by gamma irradiation. We exploited this sensitivity to screen for dominant suppressors of this phenotype by introduction of a lentiviral library containing 17,500 full-length human cDNAs. We irradiated HCC1937 cells with incremental doses ranging from 1.0 Gray to 3.0 Gray allowing 48 hours for recovery. After seven rounds of such irradiations, we pooled all the surviving cells and extracted DNA from the pooled population.

By PCR amplification using T3 and T7 primers, which flank the cDNA insert, we identified 6 distinct bands ranging in size from approximately 0.7 kbp to 2.8 kbp (Figure 1). Control HCC1937 cells that were not infected with the library yielded no products. We have cloned 4 of these bands into an expression lentiviral vector and individually transduced them into HCC1937 cells. All four clones conferred irradiation resistance to parental cells to similar extent as the wild-type BRCA1 gene. All clones are novel factors that have not been reported in DNA damage repair pathways. Currently, we are characterizing the mechanism of these clones in substituting BRCA1 in conferring resistance to DNA damage.

Based on our initial screening, we have identified a number of candidates that are involved in DNA damage repair pathway mediated by BRCA1, which is an important aspect of tumor suppression of the molecule. As none of the candidates previously has been reported to play a role in DNA damage repair pathway, we speculate that some novel components, such as RNA or chromatin structure, might be involved. Once verified and fully investigated, the possible target genes will contribute to the understanding of the biology of inherited breast cancer as well as the diagnosis and treatment of breast, ovarian, and possibly other cancers.

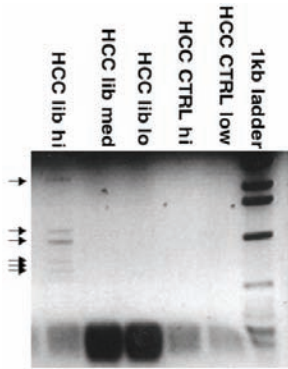
This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0252.

P67-5: ROLE OF COFACTOR OF BRCA1 (COBRA1) IN MAMMARY TUMORIGENESIS

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Breast cancer 1 (BRCA1) is a tumor suppressor gene for hereditary breast and ovarian cancers. The BRCA1 protein interacts with numerous protein partners that are involved in multiple cellular functions such as transcriptional regulation and DNA damage responses. Elucidation of the functional significance of these interactions is key to the understanding of BRCA1's role in tumorigenesis of the breast and ovarian tissues. Cofactor of BRCA1 (COBRA1) is first identified as a BRCA1-binding protein. Subsequent studies demonstrate that COBRA1 is an integral subunit of the negative elongation factor complex (NELF) and serves as a corepressor of estrogen receptor α (ER α) through its inhibitory effect on RNA polymerase II-mediated transcription elongation. In line with the physical interaction between COBRA1 and BRCA1 and their functional similarity in transcriptional regulation, a genome-wide microarray study indeed indicated a concerted action of COBRA1 and BRCA1 in regulating a common group of target genes in breast cancer cells. To ascertain the role of COBRA1 in mammary tumorigenesis, we examined COBRA1 expression in clinical samples from both tumor and normal tissues. We found that COBRA1 expression was inversely correlated with breast cancer progression, as samples of patients who had distant metastasis and local recurrence expressed very low levels of COBRA1 when compared to those who were disease free for over 10 years ($p=0.0065$ and 0.0081 , respectively). To discriminate a causal or bystander effect of COBRA1 reduction on breast cancer progression, we carried out a xenograft study by comparing tumor growth of control or COBRA1 knockdown ZR-75-1 cells in athymic mice. Tumors from COBRA1-depleted cells, but not the control cells, grew continuously in the absence of exogenously added estrogen, whereas no significant difference was observed when estrogen was supplemented exogenously. In addition, COBRA1 knockdown tumors had a higher proportion of Ki67-positive cells. The in vivo growth advantage of the COBRA1-knockdown cells at a low endogenous estrogen concentration was consistent with the higher sensitivity of the same cells to suboptimal concentrations of estrogen in DNA synthesis in vitro. In summary, our in vivo study provides a functional link between regulation of transcrip-



The PCR products of the pooled surviving HCC1937 cells

tion elongation, cancer cell proliferation, and mammary tumor growth. Currently, we are investigating the underlying molecular mechanisms as to COBRA1's function in mammary tumorigenesis. Furthermore, it will be of great interest to explore the functional cooperation of COBRA1 and BRCA1 in suppressing tumor initiation and progression in breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0302 and National Institutes of Health.

P67-6: A NOVEL ROLE FOR BRCA1 IN THE MITOTIC SPINDLE ASSEMBLY

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Hereditary inactivating mutations in the *BRCA1* tumor suppressor gene confer high risk of breast and ovarian cancer. In vivo, most of the BRCA1 protein exists in a complex with a structural related partner, BARD1. The BRCA1/BARD1 heterodimer exhibits an E3 ubiquitin ligase activity whose physiological substrates remain elusive. It is widely believed, although not convincingly proven, that the loss of BRCA1 function in DNA damage response and repair by homologous recombination is the primary cause of chromosomal instability and tumorigenesis in BRCA1 patients.

To study the evolutionary conserved function of BRCA1/BARD1 in the maintenance of genome integrity, we employ frog (*Xenopus laevis*) cell free egg extracts, which recapitulate essential cellular processes and allow to overcome the problem of non-viability of BRCA1^{-/-} cells. Our study revealed that in both *Xenopus* egg extracts and mammalian cells BRCA1/BARD1 is required for proper mitotic spindle pole assembly and for the accumulation of TPX2, the microtubule nucleating and spindle pole-organizing protein, on spindle poles (Figure 1). This function depends upon both the BRCA1/BARD1 E3 ubiquitin ligase activity and the integrity of the leucine zipper motif of the TPX2 partner, XRHAMM. BRCA1/BARD1 forms endogenous complexes with TPX2, NuMa, and XRHAMM, and it specifically attenuates XRHAMM function. By implicating BRCA1/BARD1 in the regulation of RHAMM, TPX2, and Aurora A, which are overexpressed or amplified in certain cancers, our study pointed to the existence of a tumor suppressor-oncoprotein network that operates during mitotic spindle assembly (Figure 2).

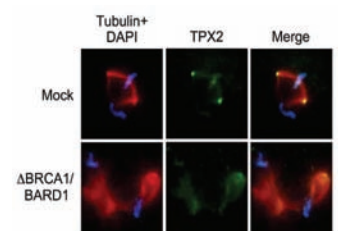


Figure 1. BRCA1/BARD1 is required for spindle pole assembly and TPX2 accumulation on spindle poles. Microtubule asters induced by chromatin in the mock-treated and BRCA1/BARD1-depleted metaphase-arrested extracts supplemented with rhodamine-labeled tubulin and Alexa Fluor 488-labeled anti-TPX2 antibody.

Recently, we have purified and identified a spindle pole protein, which is ubiquitinated in a BRCA1/BARD1-dependent manner. We are now analyzing how the aforementioned ubiquitination relates to the mitotic function of the BRCA1/BARD1 ubiquitin ligase.

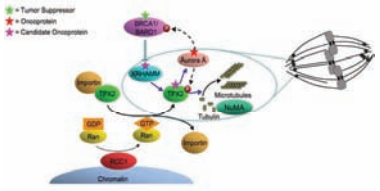


Figure 2. Schematic representation of BRCA1/BARD1 function during mitotic spindle assembly

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0524.

P67-7: SPONTANEOUS TUMOR DEVELOPMENT IN MICE CARRYING A TARGETED MUTATION OF THE Chk2 PHOSPHORYLATION SITE IN BRCA1

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Germline mutations of BRCA1 predispose women to breast and ovarian cancers. Tumor occurrence in BRCA1 mutant carriers is often associated with loss of the wild-type allele of BRCA1, suggesting that BRCA1 functions as a tumor suppressor. Consistent with the notion, studies based on cell culture models strongly indicate that BRCA1 is involved in DNA repair, cell cycle checkpoint control, and maintaining genome stability. Attempts have been made over the last decade to establish mouse models to provide direct in vivo evidence on BRCA1 functions. However, Brca1-related mouse models have so far provided limited insights on the role of BRCA1 in tumorigenesis, mainly due to the following two reasons: (1) Unlike humans, mice carrying a mutant

Brcal allele do not have an elevated risk for tumor occurrence and (2) mice homozygous for the mutant Brcal allele are embryonic lethal. To circumvent the embryonic lethal problem, most Brcal-related mouse models to date were generated on mutant genetic background such as tumor suppressor p53 or p53^{-/-}. While still informative, this approach is less than ideal since p53 mutation might significantly alter the tumorigenic pathways initiated by BRCA1 mutation alone. BrcalS971A/S971A mouse model was generated by gene targeting that carries a single mutation in Brcal that substitutes Serine971 with Alanine (S971A). Serine971 in mouse Brcal corresponds to Serine988 in human BRCA1, which has been shown in cell culture to be phosphorylated by Chk2 upon DNA damage. Unlike other Brcal mutants, BrcalS971A/S971A mice are viable without a developmental defect. In its original mixed genetic background (129SvEv and FVB/N), BrcalS971A/S971A mice are generally tumor free, with the majority of females developing uterus hyperplasia and ovarian abnormalities by 2 years of age. We report here that, in the background of mixed 129SvEv and FVB/N and C57BL/6, 4% of the heterozygous and 20% of the homozygous mutant mice, both male and female, developed tumors in multiple tissues at the ages of 4 to 6 months. Although the tumor spectrum in this Brcal mouse model differs from humans, in which loss of BRCA1 function predominantly leads to breast and ovarian cancers, our mouse model provides direct in vivo evidence that BRCA1 mutation plays an important role in initiating tumorigenesis. Currently, we are studying the impact of the BrcalS971A mutation on DNA repair and other cellular functions using tumor and normal cells derived from BrcalS971A/S971A mice. Results will be discussed. In this study, we report that a mouse model carrying a germline mutation in Brcal develops spontaneous tumors in multiple tissues, clearly demonstrating that BRCA1 is a tumor suppressor. Furthermore, similar to human pathology, tumors also occur in heterozygous mutant mice. Mechanistic studies using this in vivo model will provide crucial insights on how loss of BRCA1 plays a role in tumor initiation and progression. In addition, this animal model could be potentially suitable for developing drugs for BRCA1-related cancer prevention.

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P67-8: THE RELATIONSHIP OF BRCA1 TO REPLICATION OF PERICENTRIC HETEROCHROMATIN AND ITS IMPLICATIONS FOR BROAD EPIGENOMIC INSTABILITY IN CANCER

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Background and Objectives: More than 10 years after identifying BRCA1 as a key gene in hereditary breast cancer, fundamental aspects of BRCA1 function are still unresolved. While BRCA1 foci form at sites of induced DNA damage linked to repair, any significance of BRCA1 foci that are consistently present in normal cells has remained unknown. Several studies report loss of the inactive X chromosome in aggressive breast or ovarian cancers, and one study reported that BRCA1 normally localizes with XIST RNA on the inactive X (Xi). This implicated BRCA1 in maintenance of Xi heterochromatin in normal cells, suggesting a mechanistic link to these predominantly female cancers. Thus, we further investigated the relationship of BRCA1 to XIST RNA and X-inactivation but discovered its relationship to heterochromatin more broadly.

Methods: Molecular cytological procedures, analyzed by 2D and 3D digital imaging, were used to examine BRCA1 in relation to XIST RNA and the Xi. We also examined effects of a dox-inducible BRCA1 gene in BRCA1(-) breast cancer cells, or RNAi to BRCA1 in normal cells. Markers of Xi were assessed, as was the peripheral heterochromatic nuclear compartment, by hybridization to hnRNA. PCNA or BrdU detected replicating DNA.

Results: Findings confirm frequent Xi defects in breast cancer lines, via mitotic loss of Xi or effects on XIST RNA expression or localization. However, this did not strictly correlate with BRCA1 status. While induced BRCA1 over-expression enhanced XIST expression in some BRCA1(-) cancer cells, results indicate that broad epigenetic changes in cancer contribute to compromised Xi heterochromatin and XIST mis-regulation. BRCA1 associates with part of Xi in a subset of normal cells but does not substantially co-localize with XIST RNA to "paint" the Xi; rather a smaller focus of BRCA1 abuts the Xi. This is a key difference from other proteins involved in X-inactivation, which coat most of Xi. Interestingly, although BRCA1 localization does not simply mirror that of replicating DNA, BRCA1 foci often abut replication foci in mid-to-late S phase. Further analysis shows that discrete BRCA1 foci in human and mouse cells overwhelmingly localize to the heterochromatic nuclear compartment and many abut markers of interphase centromeres. In mouse cells BRCA1 coats blocks of pericentric heterochromatin (PCH), in a manner closely correlated with its replication. In addition, Topoisomerase II (linked to BRCA1) also localizes to these blocks of satellite heterochromatin during their replication. BRCA1(-) cancer cells demonstrated mitotic defects including post-mitotic DNA bridges, which we find commonly contain satellite DNA. Finally, results suggest that Xi defects in breast cancer occur in the context of broad compromise of peripheral heterochromatin.

Conclusions: While our findings do not suggest a direct role for BRCA1 in XIST RNA localization, they provide the first evidence implicating BRCA1 in the replication of centric/pericentromeric heterochromatin, structures key to genomic stability. Further, Xi defects may reflect broader epigenetic changes that lead to breakdown of

peripheral heterochromatin. We propose that heterochromatic instability, along with genomic instability, is a key factor in tumorigenesis and likely has a fundamental role to the development of many breast and other cancers.

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P67-9: MAPPING MAMMARY EPITHELIAL CELL TRANSFORMATION IN BRCA1 MUTANT MICE

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Recently developed culture models provide us with a window into the initial stages of breast cancer formation when some molecular changes have already occurred, but the typical malignant growth is not yet present. We have called these cells that have already taken one or more "hits," but do not yet form cancer, tumor progenitor cells. It is our hypothesis that the breast tissue of BRCA1 carriers harbors a population of cancer progenitor cells that can be identified with suitable culture techniques. We used a BRCA1 mutant mouse model to address this question. The BRCA1 mutant mice were bred and their breast tissue cells systematically analyzed in an assay that allows for the growth of these cells in three dimensions where their growth pattern most closely resembles the mammary gland environment. Culture conditions for the growth of these mammary epithelial cells were established; that is, growth in a 2% matrigel medium supplemented with EGF, insulin, and hydrocortisone. Conditional BRCA1 mutant mice were bred together with the respective controls.

At various stages of mammary gland development, these mice were sacrificed and their mammary glands histologically examined and plated in the newly developed colony formation assay. These studies were performed with mammary glands that had not yet developed tumors to assess the changes in a mammary epithelium "at risk" that has not yet undergone transformation.

The number of colonies derived from the primary mammary epithelium of these mice at the ages of 6 and 9 months was not significantly different. We did, however, observe that the diameter of the colonies was larger in the BRCA1 null PMECs. We are therefore currently examining these colonies with specialized image analysis software. We have, as originally planned, created a tissue bank with fixed paraffin-embedded tissues. We then decided to introduce a second mutation that is often found early on in BRCA1-related cancers, p53 heterozygosity. We found that cancer-like colonies are found if BRCA1-null primary mammary epithelial cells are also p53 heterozygote. We conclude that at least in mice, an additional genetic hit may be required for PMECs to show features of cancerization in ex vivo cultures, such as heterozygosity for p53.

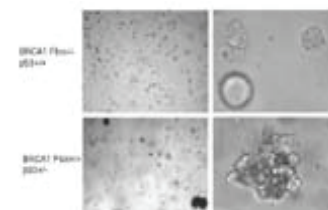


Figure 1: Colonies derived from primary mammary epithelial cells (PMECs) in BRCA1-null mice (upper panel) and in BRCA1-null PMECs that are also p53 heterozygotes (lower panel). Left 4x, right 20x magnifications.

We found that cancer-like colonies are found if BRCA1-null primary mammary epithelial cells are also p53 heterozygote. We conclude that at least in mice, an additional genetic hit may be required for PMECs to show features of cancerization in ex vivo cultures, such as heterozygosity for p53.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0453.

P67-10: REGULATION OF BRCA1 EXPRESSION BY Sam68

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Background: Sam68 is a substrate for c-Src and BRK/Sik (breast tumor kinase) kinases. BRK/Sik kinase, which is expressed at high levels in breast tumor cell lines, was shown to phosphorylate Sam68 and negatively regulate its function. Although its cellular function is enigmatic, Sam68 was implicated in the nuclear export of HIV-1 RNAs. Since BRK/Sik kinase negatively regulates Sam68 function, our goal was to test whether Sam68 is involved in the export/stability of BRCA1 mRNA. Patients who have either lost or have mutations in tumor suppressor genes BRCA1 or BRCA2 are at high risk of developing breast cancers. We reasoned that mutant Sam68 may not recognize the mutant BRCA1 mRNAs, and thus, failing to be exported to the cytoplasm, which in turn, may be resulting in low levels of BRCA1 expression.

Objectives: Our objectives were (1) elucidate the role of Sam68 in BRCA1 mRNA export and (2) delineate the BRCA1 mRNA export pathway.

Methods: For objective 1, we used an RNAi strategy to knockdown Sam68 followed by western blot analysis. We tested the Sam68 depletion by western analysis and the effect of the knockdown of Sam68 on BRCA1 by northern analysis using BRCA1³²P-labeled cDNA and/or by the real-time quantitative RNA PCR using BRCA1 primers. In addition, we tested the effect of transdominant negative mutant of Sam68 on BRCA1 expression by western analysis using anti-BRCA1 antibodies. For objective 2, we

treated cells with leptomycin B (LMB) and compared the levels of total BRCA1 RNA to the cytoplasmic RNA by northern and PCR analyses.

Results: To accomplish objective 1, we knocked down Sam68 in MCF7 cells and demonstrated that Sam68 knockdown cells express reduced levels of BRCA1 mRNA judged by northern and quantitative RT-PCR analyses. Additionally, we showed that the cells expressing mutant Sam68 had reduced levels of BRCA1 protein. These results suggest that Sam68 is involved in BRCA1 expression. To map the RNA export pathway of BRCA1 (objective 2), we treated MCF7 cells with LMB drug and analyzed the BRCA1 RNA levels in total and cytoplasmic fractions. In LMB-treated cells, the RNA exported to the cytoplasm was far less compared to the total RNA, suggesting that BRCA1 uses the CRM1-dependent RNA export pathway.

Conclusions: From our results, it is evident that Sam68 positively regulates BRCA1 expression. Although our results do not pinpoint a step in BRCA1 RNA metabolism, it is our expectation that Sam68 depletion might affect the BRCA1 RNA stability. Alternatively, the effect of Sam68 could be at the level of BRCA1 transcription. Our LMB results suggest that BRCA1 uses CRM1-dependent RNA export way. Further work is needed to map the specific step for Sam68 in BRCA1 regulation. When we succeed in doing so, our plan will be to identify pharmacological agents that upregulate Sam68 in a nonstressful manner for preventive and therapeutic purposes of breast cancer. In summary, it is important to continue to support the applications dealing with RNA export mechanisms as they shed light on the fundamental aspects of cellular RNA transport mechanisms, which in turn may provide insights into the development of novel strategies and a new class of drugs to fight breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0529 and National Institute of Allergy and Infectious Diseases.

P67-11: TUMOR SUPPRESSION BY BRCA1: A CRITICAL ROLE AT DNA REPLICATION FORKS?

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Columbia University College of Physicians and Surgeons

Background and Objectives: Mutations in the BRCA1 tumor suppressor gene occur in about 40% of familial cases of breast cancer. BRCA1 is involved in various cellular processes including DNA repair, transcriptional regulation, chromatin remodeling, and cell cycle checkpoint regulation. ATM phosphorylates BRCA1 in response to double-strand breaks (DSBs) and ATR in response to replication inhibition. Furthermore, BRCA1/BARD1 nuclear foci are observed in untreated S-phase cells, supporting a role for BRCA1 and BARD1 in DNA replication. In addition, BRCA1/BARD1 deficient cells are hypersensitive to cross-linking agents such as cisplatin and mitomycin C and double-strand breaks formation. The major objectives of this study were: (1) To investigate BRCA1/BARD1 association with the replicating fork. (2) To investigate the behavior role of BRCA1/BARD1 when a replication fork encounters an interstrand DNA cross-link (ICL) and determine the potential role of the complex in signaling and repair of ICLs.

Methodologies: We used cell-free extracts derived from the eggs of *Xenopus laevis* to investigate the role of BRCA1/BARD1 complexes in DNA replication and DNA repair of ICLs. These extracts allow studying the role of proteins that are essential for growth, such as BRCA1. Furthermore, these extracts faithfully support DNA replication and many aspects of the DNA damage response including DNA repair. We have also designed a DNA substrate that is a model for the study of ICLs generated by platinum compounds, routinely used in cancer chemotherapy, including cases of breast cancer. Studies addressing specifically the repair of ICLs have been very limited mostly due to the heterogeneous nature of the lesions arising from cross-linking agents. To overcome this obstacle, we have generated a plasmid DNA containing a single, defined ICL.

Results: (1) We show that BRCA1 and BARD1 associate with chromatin. BRCA1 association to chromatin is replication-dependent and requires the formation of a pre-Replicative Complex (pre-RC) as Geminin, which inhibits MCM chromatin loading, can block it. In contrast, Aphidicolin, an inhibitor of DNA polymerases does not prevent BRCA1 from binding to chromatin. These results suggest that BRCA1/BARD1 complex could be a component of the replication fork, even in the absence of DNA damage. (2) We show that a DNA template containing a single ICL is replicated and repaired in *Xenopus* cell free extract. This establishes *Xenopus* extracts as a unique system to study the signaling from ICLs as well as their repair. We observe that replication through the DNA adduct can take place in a DNA replication dependent and independent manner. Furthermore, we show that whereas acute depletion of BRCA1 has modest consequences on ICL repair, it affects significantly the DNA damage response signaling triggered by the ICL.

Conclusions: We have established a system to study the precise role of BRCA1 in DNA replication and in DNA replication fork stability. Furthermore, we show that BRCA1 is implicated in signaling from ICL. Since ICL is thought to be the lesion primarily responsible for the toxicity of cross-linking platinum compounds used in chemotherapy, our study provides the first mechanistic bases to explain the exquisite sensitivity of BRCA-deficient cells to cross-linking agents.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0494.

P67-12: MECHANISMS OF SILENCING AND DESILENCING OF BRCA2 GENE EXPRESSION IN HUMAN BREAST CELLS

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Meharry Medical College, Nashville

The tumor suppressor protein BRCA2 is essential for DNA recombination repair as well as the regulated and synchronized growth of human breast cells in the developing breast tissues. Its expression is strictly regulated depending upon the growth stage of the breast cell. It is not expressed in the nondividing human breast cells, and it is only expressed in the dividing stages of the cells. The hypothesis is that balanced expression of BRCA2 in the rapidly dividing human breast cells is critical to prevent the malignant transformation of these cells. We describe here our findings of an epigenetic mechanism of human BRCA2 gene expression regulation through an Alu repeat and E2-box (CACCTG/CAGGTG)-containing transcriptional silencer located at the promoter of this gene. In this mechanism, the transcriptional repressor protein SLUG binds to the silencer in non-dividing human breast cells to inhibit the expression of this gene by chromatin remodeling via the recruitments of CtBP1 and HDAC1 and histone deacetylation as was established by chromatin immunoprecipitation and reporter gene expression analyses. Although alanine-substitution mutagenesis revealed that the putative CtBP1 binding site (PSDTSSK) in human SLUG protein is essential for its suppressor activity, yeast-2-hybrid and co-IP analysis showed that it does not directly binds to CtBP1 protein. We are in the process of developing peptide aptamers against this SLUG-specific essential PSDTSSK site to test whether these aptamers can manipulate SLUG function and thus BRCA2 gene expression in human breast cells. In the dividing breast cells, the DNA binding nuclear isotype of peroxiredoxin 5 (PRDX5) apparently desilences BRCA2 gene expression by competing with the binding of SLUG to the silencer. Our data also suggest that, as estrogen and the members of the P53 family of proteins regulate SLUG gene expression, they may also cell cycle dependently regulate the expression of BRCA2 gene in human breast cells perhaps by regulating SLUG gene expression. Understanding the molecular mechanisms involved in the silencing/desilencing of the expression of the BRCA2 gene in the human breast cells is important because any perturbation or malfunction of these mechanisms will result in the dividing cells lacking appropriate levels of BRCA2 at "the time of need" during cell cycle and thus will predispose these cells to malignancy. If we understand the silencing/desilencing mechanisms in detail, we should be able to effectively intervene in the pathways and thus be able to efficiently combat the onset of human breast cell malignancy caused by these malfunctions.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0341.

P67-13: A FACS ASSAY FOR E3 UBIQUITIN LIGASE ACTIVITY AS A MODEL TO STUDY BRCA1 IN HUMAN BREAST CANCER

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Background: Breast cancer is the second leading cause of cancer deaths in women in the United States. It is estimated that between 5%–10% of breast cancers have a heritable basis, and mutations in breast cancer susceptibility gene 1 (BRCA1) gene contribute significantly to the development of these breast cancers. BRCA1 is a tumor suppressor protein that inhibits the uncontrolled proliferation of cells, in part, by promoting the destruction of other cellular proteins in its role as an E3 ubiquitin (Ub) ligase. Current methods for monitoring the activity of E3 Ub ligases in cell culture or in vivo have been limited. As a result, the degradation of known cellular targets by many E3 Ub ligases, including BRCA1, in live cells has not yet been examined. Without this knowledge, it will not be possible to understand how the progression of breast cancer is regulated to facilitate its detection and treatment.

Objective: The objective of our research is to develop a strategy to monitor the activity of E3 Ub ligases against known targets relating this activity of BRCA1 to its anti-proliferative activity. Our rationale for these studies is that by identifying and characterizing the molecular events that control the proliferation of breast cells, it may be possible to develop new therapies for preventing or treating breast cancer.

Methods: To monitor the activity of E3 Ub ligases including BRCA1, proven targets of these ligases will be expressed as fluorescently labeled proteins in cell culture. If an E3 Ub ligase mediates the degradation of an established target in cell culture, it is expected that the target protein will show a reduced fluorescence signal in the presence of the ligase as measured by flow cytometry. Because herpes simplex virus type 1 (HSV-1) infected cell protein 0 (ICP0) is an E3 Ub ligase and a small group of its targets have been identified, we initially used ICP0 and one of its demonstrated targets, promyelocytic leukemia (PML), in experiments to determine the feasibility of our approach.

Results: To establish proof of principle in our study, mammalian cells that express a PML-GFP fusion protein were selected by cell sorting and infected with an adenovirus vector that expresses ICP0. ICP0 E3 Ub ligase activity was examined in these infected cells by fluorescence microscopy and FACS analysis for a reduction in their fluorescence. In contrast to uninfected cells, only PML-GFP-expressing cells infected with the

ICP0 adenovirus vector led to a significant decrease in the fluorescence signal of PML-GFP by both fluorescence microscopy and FACS analysis.

Conclusions: Using HSV-1 ICP0 as a paradigm, it is possible to examine the activity of an E3 Ub ligase (via one of its known targets) in cell culture with FAC analysis. Future work will involve monitoring the E3 Ub ligase activities of wild-type and mutant forms of BRCA1, correlating these activities with their tumor suppressor capabilities.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0365 and National Center for Research Resources (P20 RR015563).

P67-14: TARGETING BRCA1 TO DNA DAMAGE SITES

Xiaochun Yu

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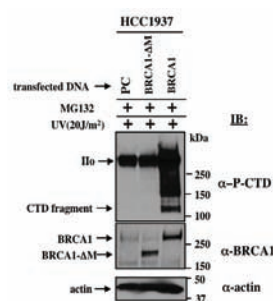
Breast cancer susceptibility gene 1 (BRCA1) participates in DNA damage response. However, following DNA damage, the mechanism by which BRCA1 is recruited to DNA damage sites is remaining elusive. Recently, we have demonstrated two new BRCA1 partners, RAP80 and CCDC98. BRCA1, CCDC98, and RAP80 form a complex. BRCA1 BRCT domain directly recognizes phospho-Ser406 of CCDC98. CCDC98 mediates BRCA1's association with RAP80. Moreover, both RAP80 and CCDC98 control DNA damage-induced BRCA1 translocation and accumulation at DNA damage sites and BRCA1-dependent G2/M checkpoint activity. Taken together, our results demonstrate RAP80 and CCDC98 target BRCA1 to DNA damage sites and mediate BRCA1-dependent DNA damage response.

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P67-15: BRCA1 MEDIATES CLEAVAGE OF RNAPII WITHIN A HIGHLY CONSERVED TRANSCRIPTION-DEPENDENT DAMAGE SURVEILLANCE PATHWAY

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BRCA1 has been implicated in numerous DNA repair pathways that maintain genome integrity; however, the function responsible for its tumor suppressor activity in breast cancer has remained obscure. To identify the most conserved and least dispensable of the many BRCA1 interactive functions, we utilized functional genomic screens in *Saccharomyces cerevisiae* to identify mutants that suppressed the G1 checkpoint arrest and lethality induced following heterologous BRCA1 expression. A genome-wide screen in the yeast diploid deletion collection combined with a screen of ionizing radiation-sensitive gene deletions identified mutants that permit growth in the presence of BRCA1. These genes delineate a metabolic mRNA pathway that temporally links transcription elongation (*SPT4*, *SPT5*, *CTK1*, *DEF1*) to nucleopore-mediated mRNA export (*ASM4*, *MLP1*, *MLP2*, *NUP2*, *NUP53*, *NUP120*, *NUP133*, *NUP170*, *NUP188*, *POM34*) and cytoplasmic mRNA decay at P-bodies (*CCR4*, *DHH1*). Strikingly, BRCA1 interacted with the phosphorylated RNA polymerase II (RNAPII) carboxy terminal domain (P-CTD), phosphorylated in the pattern specified by the CTDK-I kinase, to induce DEF1-dependent cleavage and accumulation of a RNAPII fragment containing the P-CTD. Significantly, breast cancer associated BRCT domain defects in BRCA1 that suppressed P-CTD cleavage and lethality in yeast also suppressed the physical interaction of BRCA1 with human SPT5 in breast epithelial cells, thus confirming SPT5 as a relevant target of BRCA1 interaction. Furthermore, enhanced P-CTD cleavage was observed in both yeast and human breast cells following UV-irradiation indicating a conserved eukaryotic damage response. Moreover, P-CTD cleavage in breast epithelial cells was BRCA1-dependent since UV-induced P-CTD cleavage was only observed in the BRCA1-BRCT domain mutant cell line HCC1937 following ectopic expression of full-length wild-type BRCA1 and treatment with the proteasome inhibitor MG132 (Fig. 1; PLoS One in press). Cells expressing an internally deleted, less toxic form of BRCA1 (BRCA1-delta M) that retained functional RING and BRCT domains, failed to demonstrate UV-induced P-CTD cleavage (Figure 1). Finally, BRCA1, SPT5, and hyperphosphorylated RPB1 form a complex that was rapidly degraded following MMS treatment in wild-type but not BRCA1-mutant breast cells. Combined, these results suggest that BRCA1 interacts with RNAPII in a DNA damage surveillance function within actively transcribing genes. This extends the mechanistic link between BRCA1 and transcriptional



consequences in response to DNA damage and suggests an important role for RNAPII P-CTD cleavage in BRCA1-mediated cancer suppression.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0232; Italian Association for Research on Cancer; and National Institutes of Health (CA84955).

P67-16: ABSTRACT WITHDRAWN

P67-17: ROLES OF BRCA1 IN MAMMARY HOMEOSTASIS

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Background and Objectives: Mutations in the breast cancer susceptibility gene BRCA1 account for up to half the hereditary breast cancer cases and almost all the hereditary breast and ovarian cancer cases. Also, BRCA1 expression is often decreased during sporadic breast cancer progression. Although a tissue-specific role of BRCA1 is implicated, the cumulated evidence mainly converges on its global functions to maintain the genomic stability. To address specific functions of BRCA1 in breast, we hypothesized that BRCA1 serves to maintain the normal mammary epithelia and tested its roles in differentiation of mammary epithelial cells (MECs) as well as regulation of the surrounding tissue microenvironment.

Methodologies: We depleted BRCA1 in nonmalignant MCF10A cells with adenoviral RNAi and tested their acinus differentiation in 3-D culture. We ectopically expressed RNAi-resistant BRCA1 carrying cancer-linked mutations and tested a resumption of acinus formation. The gene expression profile of these cells was compared with that of control cells using microarray. Another microarray analysis was performed on cells depleted of CtIP, a BRCA1-associated transcriptional co-repressor. Among a group of genes co-repressed by BRCA1 and CtIP, angiopoietin-1 (ANG1), a secreted angiogenic factor, was further studied for the BRCA1/CtIP-mediated transcriptional repression on its promoter using reporter assays. The region of ANG1 promoter responsible for this repression was tested for a direct binding by ZBRK1, a BRCA1-associated transcriptional repressor, using EMSA and ChIP analyses. MCF10A cells depleted of either BRCA1 or CtIP by adenoviral RNAi were co-cultured with endothelial cells in 3-D matrix and tested for the induction of angiogenesis. Finally, BRCA1-/-;p53-/- mouse mammary tumors were compared with p53-/- tumors and analyzed for the tumor growth rate and degree of vascularization.

Results: We found that BRCA1-depleted MECs failed to form defined acinus structures in 3-D matrix but instead grew into an irregular-shaped large cluster with six-fold higher proliferative potential compared to control cells. Such an aberration was rescued by the ectopic expression of RNAi-resistant BRCA1 carrying wild-type sequence or Q356R mutation but not by BRCA1 with M1775R mutation in the C-terminal BRCT domain. We analyzed the gene expression profiles of BRCA1-depleted cells in 3-D culture, as well as cells depleted of CtIP, a transcriptional co-repressor associated with the BRCT domain of BRCA1. We identified a dozen genes co-repressed by BRCA1 and CtIP. We further studied ANG1, one of the co-repressed genes, for the mechanism of repression. BRCA1 and CtIP in concert with ZBRK1, a BRCA1-associated zinc-finger protein, formed a transcriptional repressor complex at the ANG1 promoter via a ZBRK1 binding site. A loss of either protein derepressed ANG1 expression from MECs, which in turn stabilized adjacent endothelial cells in 3D co-culture. Furthermore, ANG1 derepression contributed to the accelerated growth and enhanced vascularization of BRCA1-deficient mouse mammary tumors.

Conclusion: Our study demonstrates that BRCA1 functions to promote differentiation of MECs and to prevent angiogenesis of the surrounding microenvironment. Thus, a loss of BRCA1 impairs the homeostasis of mammary epithelia and serves as both an intrinsic and extrinsic pathogenic drive for neoplastic growth.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0322.

P67-18: BREAST CANCER SUSCEPTIBILITY GENE 1 SUPPRESSES PROGESTERONE STEROID HORMONE RECEPTORS

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Formation of breast cancers is subjected to regulation by several hormones. Progesterone, an ovarian steroid hormone, choreographs reproductive events - ovulation, luteinization, and mammary gland development; its deregulation leads to breast carcinogenesis. In response to progesterone, progesterone receptor (PR)—a member of the nuclear/intracellular receptor superfamily—translocates from nuclear membrane into nucleus and functions as a transcription factor regulating progesterone responsive genes expression.

Several isoforms of PR have been observed; major ones are PR-A (94 kDa) and PR-B (116 kDa). In *PR-A* knockout (PRAKO) mice, uterine and ovarian function abnormally but responses to progesterone in mammary gland remain normal. On the other hand, distorted mammary gland morphogenesis during pregnancy develops in *PR-B* knockout (PRBKO) mice; in contrast, depletion of PR-B isoform does not affect progesterone responses of the ovary or uterus. Nevertheless, molecular regulators altering PRs expression and activity have received particular scrutiny.

BRCA1, identified as a breast cancer susceptibility gene based on genetic linkage analyses, has been reported as a key player in many cellular processes including transcription, DNA repair, check point activation, and protein degradation. Its germline or somatic mutations may influence women to form breast cancers and ovarian cancers. Regardless of tissue specific functions of *PR-A* and *PR-B* during development, both forms are up-regulated in *BRCA1* mutated breast cancers. However, it has remained elusive whether inhibitory effects of BRCA1 on PR expression and activity are mediated through PR or coactivators degradation or transcriptional repression.

In *BRCA1*-deficient mouse mammary gland, PRs expression increased, suggesting that *BRCA1* may exert negative effects on PRs expression. To further investigate on the control of the PR proteins expression by BRCA1, amount of PRs was quantitatively analyzed in proteasome inhibitor treated *p53*-mutated and *p53/BRCA1*-deficient mammary epithelial cells. While PR amount was restored in *p53*-deficient cells after proteasome-inhibitor treatment, expression level of PRs remained the same in *p53/BRCA1*-

deficient cells, indicating that BRCA1 may negatively regulate PRs expression through proteasome pathway. To this end, in vitro ubiquitination assay will be performed to examine whether PRs are direct substrate of BRCA1 in the present study.

Downstream target genes of PRs concert complex of reproductive events. Since inhibition of PRs activities by BRCA1 has been documented under in vitro condition, it will be of greatest interest to examine how BRCA1 regulates PRs activity in vivo especially during chronic treatment of progesterone, where the in vitro system cannot provide such physiological information. Recently, Progesterone Receptor Activity Indicator (PRAI) transgenic mouse model system has been established to monitor transcriptional activity of PRs in vivo. Using Upstream Activating Sequences (UAS) for the Gal4 gene driven green fluorescent protein (GFP) as a readout, the PRAI system harbored engineered PRs where its DNA binding domain was deleted and replaced with the Gal4 DNA binding domain. Thus, the activities of PRs can be demonstrated qualitatively by in situ hybridization or quantitatively by fluorescent intensity measurement.

To gain more insights into hormonal actions interplayed by PRs at physiological level, bigenic *PRAI-BRCA1^{f1/f1}* mice has been generated to see if BRCA1 exerts any effects on transcriptional networks coordinated by PRs. These mechanistic studies will advance knowledge in breast cancers and help find possible targets for cancer prevention.

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ONCOGENES II

Poster Session P68

P68-1: TEL-NTRK3 FUSION ONCOGENE INITIATES BREAST CANCER FROM COMMITTED MAMMARY PROGENITORS VIA ACTIVATION OF AP1 COMPLEX

Stuart Orkin,¹ Zhe Li,¹ Cristina Tognon,² Frank Godinho,¹ Laura Yasaitis,¹ Hanno Hock,³ Jason Herschkowitz,⁴ Chris Lannon,² Eunah Cho,⁵ Seong-Jin Kim,⁵ Roderick Bronson,⁶ Charles Perou,⁴ and Poul Sorensen²

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Background and Objectives: Breast cancer will affect one in nine women. The majority of breast cancer cases are sporadic, yet their mechanisms of oncogenesis remain largely undefined in part due to their cytogenetic complexity, which has largely precluded the discovery of primary genetic events that lead to transformation of breast epithelium. Several years ago, a recurrent translocation involving human chromosomes 12 and 15 was identified in patients with secretory breast carcinoma (SBC). This translocation brings together coding regions of the *TEL* (also known as *ETV6*, located on chromosome 12) and *NTRK3* (also called *TRKC*, located on chromosome 15) genes such that *TEL* contributes an oligomerization domain (the PNT domain) to the protein tyrosine kinase (PTK) domain of *NTRK3*. The occurrence of such a chromosome translocation as the sole cytogenetic alteration in SBC provides an excellent opportunity to study pathways underlying the initiation and progression of breast cancer and how these pathways may be targeted therapeutically. The purpose of this research is to generate mice that faithfully express the *TEL-NTRK3* fusion gene specifically in their mammary epithelial cells as a means of creating a novel mouse model of human breast cancer. Such a model (based on the initiating genetic event in one type of human breast cancer) will enhance our understanding of cellular pathways affected in breast cancer and provide a new system for preclinical testing of potential new therapies.

Methodologies: By gene targeting in mouse ES cells, we created a conditionally expressed allele of the *TEL-NTRK3* fusion gene within the endogenous *Tel* locus (designated *lox-STOP-TN*, will refer to as *TN* from here on). We generated germline transmitting chimeras and established a colony of *TN* mice. We then specifically activated expression of the *TN* oncogene in mammary epithelial cells by breeding of *TN* mice to a mouse strain that expresses Cre-recombinase under the control of the Whey Acidic Protein (*Wap*) promoter.

Results: Activation of *TN* expression in mammary epithelial cells by *Wap-Cre* leads to fully penetrant, multifocal malignant breast cancer with short latency. Pregnancies do not significantly change the latency and kinetics of tumor development. By genetic marking, we show that in nulliparous *Wap-Cre;TN* females, committed alveolar bipotent or CD61+ luminal progenitors are targets of tumorigenesis. Furthermore, *TN* transforms these otherwise transient progenitors through activation of the AP1 complex.

Conclusions: In summary, we have created mice in which an initiating event in human breast cancer, the expression of the *TEL-NTRK3* fusion protein, is recapitulated. The conditional *TN* mice (when interbred with *Wap-Cre* transgenic mice) develop multifocal breast cancer with antecedent mammary epithelial cell hyperplasia at full penetrance. We show through genetic marking that committed mammary progenitors, rather than mammary stem cells, can be direct targets of transformation. We show that activation of the AP1 complex represents a critical downstream event of the *TEL-NTRK3* translocation. Further focus on this transcriptional complex as a target in human breast cancer is warranted.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0502; National Cancer Institute; Canadian Institutes of Health Research (Canada); and Howard Hughes Medical Institute.

P68-2: A SMALL MOLECULE COMPOUND SELECTIVELY INHIBITS Akt, INCLUDING AKT1-E17K, AND TUMOR GROWTH IN CANCER CELLS WITH HYPERACTIVATED Akt

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H. Lee Moffitt Cancer Center & Research Institute at University of South Florida

The serine/threonine kinase Akt/PKB pathway is frequently hyperactivated in human cancer and functions as a cardinal nodal point for transducing extracellular and intracellular oncogenic signals, and thus presents an exciting target for molecular therapeutics. Here, we reported the identification of a small molecule Akt inhibitor, AKT-SI (*Akt* signaling inhibitor)-1. AKT-SI treatment of cancer cells results in inhibition of the kinase activities and phosphorylation levels of the three members of Akt family. In contrast, AKT-SI had no effects on the activities of the upstream Akt activators, PI3K and PDK1. Significantly, the kinase activity and phosphorylation (e.g., pT308 and pS473) levels of constitutively active Akt, including a naturally occurring mutant AKT1-E17K that resists to an allosteric Akt kinase inhibitor, were potentially inhibited by AKT-SI. AKT-SI is highly selective for Akt and does not inhibit the activation of PKC, SGK, PKA, STAT3, Erk-1/2, or JNK. The inhibition of Akt by AKT-SI resulted in induction of cell growth arrest and apoptosis selectively in human cancer cells that harbor constitutively activated Akt. Significantly, AKT-SI inhibited tumor growth in nude mice of human cancer cells in which Akt is elevated but not of those cancer cells

in which it is not. These data indicate that AKT-SI1 is an Akt pathway inhibitor with anti-tumor activity in vitro and in vivo and could be a potential anti-cancer agent for patients whose tumors express hyperactivated Akt.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0394.

P68-3: Ets TRANSCRIPTION FACTORS AND TARGET GENES IN NORMAL MAMMARY TISSUE AND TUMORS

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¹Scripps Research Institute, ²Burnham Institute, and ³Genomics Institute of the Novartis Research Foundation

The members of the Ets family of transcription factors, which share similar DNA binding specificities, are implicated in cellular transformation. Interfering with Ets transcription factor function can reverse multiple aspects of the transformed phenotype of tumor cells. The identification of the Ets factors that are causal for this Ets factor mediated reversal of cell transformation is complicated by the unknown number of Ets factors that are expressed in any cellular context. We utilized quantitative PCR assays for 25 mouse Ets factors to analyze the expression of the Ets family in normal mammary tissue, mammary-related cell lines, and mammary tumors. In normal mammary tissue, 24 Ets factors were expressed, and the most abundant Ets factor mRNAs measured were Elk4, Elf1, and Ets2. Comparison of Ets factor expression in normal mammary tissue and mammary tumors identified significantly elevated expression of Pse/PDEF, Ese2/Elf5, Ese3/Ehf, TEL/Etv6, and Elf2/NERF in mammary tumors.

To further characterize the role of Ets2 in human breast cancer cell transformation, we generated MDA-MB-435 human breast cancer cell lines stably or inducibly overexpressing Ets2. We found that MDA-MB-435 cells expressing the Ets2 DNA binding domain (Ets2DBD) exhibited a strong reduction in anchorage-independent growth, in vitro motility and invasiveness, and resistance to apoptosis. Surprisingly, cells overexpressing full-length Ets2 exhibited a similar phenotype, except that their motility was unimpaired, and xenograft tumors exhibited a delayed onset and dramatically reduced tumor angiogenesis.

We identified gene expression differences between MDA-MB-435 cells and the reverted sub-lines with altered Ets function, using cDNA microarray analysis. Identified genes include previously characterized Ets targets (e.g., MMP3, uPA, and TNC) and novel targets (e.g., S100A4, PRL-1, AKT3, PARG1, and RAB7). MDA-MB-435 cells overexpressing Ets2 upregulate the expression of Thrombospondin-1 (TSP-1). This elevated TSP-1 expression correlates with reduced vascularity seen in xenograft tumors, linking Ets2 signaling to anti-angiogenic function.

Together, this comprehensive analysis revealed unexpectedly diverse Ets family gene expression, and characterized novel Ets factor changes in mammary tumors. In addition, we described a role of Ets2 in human breast tumor cell transformation and identified a panel of target genes that correlate with Ets mediated reversion of tumor cell transformation.

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P68-4: MUC1 IS A NOVEL REGULATOR OF ErbB1 RECEPTOR TRAFFICKING

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ErbB receptors are key regulators of cell survival and growth in normal and transformed tissues. The oncogenic glycoprotein MUC1 is a binding partner and substrate for erbB1 and MUC1 expression can potentiate erbB-dependent signal transduction. After receptor activation, erbB1 is typically downregulated via an endocytic pathway that results in receptor degradation or recycling. We report here that MUC1 expression inhibits the degradation of ligand-activated erbB1. Through the use of both RNAi-mediated knockdown and overexpression constructs of MUC1, we show that MUC1 expression inhibits erbB1 degradation after ligand treatment in breast epithelial cells. This MUC1-mediated protection against erbB1 degradation can increase total cellular pools of erbB1 over time. Biotinylation of surface proteins demonstrates that cell-surface associated erbB1 receptor is protected by MUC1 against ligand-induced degradation, although this is accompanied by an increase in erbB1 internalization. The MUC1-mediated protection against degradation occurs with a decrease in EGF-stimulated ubiquitination of erbB1 and an increase in erbB1 recycling. We also show that EGFR localizes to the endocytic recycling compartment (ERC) in the presence but not in the absence of MUC1 and that MUC1 overexpressing cells associate with the adaptor protein CALM more efficiently than wild-type cells. These differences in trafficking could be essential to aberrant EGFR signaling in MUC1 overexpressing breast cancer cells. These data indicate that MUC1 expression is a potent regulator of erbB1 receptor trafficking and stability upon activation and may promote transformation through the inhibition of erbB1 degradation.

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P68-5: BREAST CANCER PHENOTYPE IS AFFECTED BY THE CELL ORIGIN

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Effects of cell differentiation stage on phenotype of oncogene-induced cancers are unknown. We therefore developed transgenic mice expressing the RCAS virus receptor TVA from the promoter of the putative progenitor cell marker Keratin 6a. Resulting TVA-expressing mammary cells were enriched for both Keratins 8 and 5, expected for progenitors. RCAS delivery of the polyoma middle T antigen oncogene generated mammary tumors in a median 14 days, suggesting that progenitor cells are highly susceptible to tumor induction. Compared with tumors induced by the same virus in more differentiated mammary cells (MMTV-TVA transgenic mice), tumors in the K6-TVA mice were more papillary, ER-negative rather than mostly ER-positive, and showed quite different gene expression profiles. Thus, phenotype is influenced by target cell differentiation.

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P68-6: GLI1 PROMOTES GROWTH, MIGRATION, AND INVASION OF BREAST EPITHELIAL CELLS

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Background and Objectives: Gli1 is a member of the *Kruppel* family of zinc finger transcription factors and a member of the hedgehog signaling pathway. Gli1 is known to be involved in tumorigenesis of several organs (i.e., prostate, pancreas, brain, and skin). Previous data from our lab indicated that Gli1 is overexpressed in breast cancer cell lines and tissues. Although Gli1 is a known oncogene and its role in cancer is implicated, the cell type-specific role of Gli1 in breast cancer has not been established.

Methods: To define the role of Gli1 in breast cancer, HA-tagged Gli1 was overexpressed by transduction of a retroviral expression vector into MCF10A cells, which are epithelial cells isolated from benign breast, and MDA-MB-231 breast cancer epithelial cells. Overexpression of Gli1 was confirmed by probing for Gli1 protein with an HA antibody by western blot analysis. To compare growth of Gli1-overexpressing MCF10A cells and vector controls, standard growth curves were performed. To evaluate the morphogenesis of MCF10A in three-dimensional (3D) growth conditions, cells were plated on solidified growth factor reduced-Matrigel with MCF10A assay media. The size of each acinar structure was measured on day 14 using image analysis software. For proliferation, the acinar structures at days 8 and 14 were immunofluorescently stained with Ki67 antibody (a proliferation marker). Migration of MCF10A and MDA-MB-231 cells overexpressing Gli1 and vector controls was measured using polycarbonate transwell filters (8 µm pore size), and invasion was measured using Matrigel-coated invasion chambers. For invasion and migration assays, the cells that migrated across the filter were stained with hematoxylin and eosin and counted. To determine downstream targets upregulated by Gli1, gene expression of MCF10A and MDA-MB-231 overexpressing Gli1 and vector controls was assessed by the Taqman Low Density Arrays (Applied Biosystems).

Results: Overexpression of Gli1 in MCF10A decreased growth in standard two-dimensional (2D) culture conditions on plastic. However, when grown in 3D, Gli1 overexpressing MCF10A formed larger ($p < 0.001$), more proliferative (%Ki67 labeling of 27% in Gli1 overexpressing MCF10A versus 15% in vector controls, $p < 0.001$), and more complicated acinar structures than the vector controls. These structures resembled preneoplastic, intraductal precursors of breast cancer. These results indicate that Gli1 induces a coordinated process that is highly context dependent and does not occur in 2D cultures. In addition, Gli1 overexpression enhanced migration and invasion of MCF10A ($p < 0.001$ and $p = 0.033$, respectively) and MDA-MB-231 ($p = 0.002$ and $p = 0.055$, respectively). Gli1 upregulates expression of some genes presumed to be involved in migration and invasion—vascular endothelial growth factor receptor/Flt1, vascular endothelial growth factor receptor 3/fms-related tyrosine kinase 4, nerve growth factor receptor, and nerve growth factor B.

Conclusions: Gli1 promotes growth and abnormal morphogenesis of MCF10A in 3D and enhances migration and invasion of both MCF10A and MDA-MB-231. These results suggest a role for Gli1 in breast carcinogenesis and cancer progression. Small-molecule inhibitors of Gli1 and hedgehog signaling have been identified, and targeting Gli1 may be effective in the treatment and prevention of breast cancer.

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P68-7: MICRORNA INHIBITORS AS ANTICANCER THERAPEUTICS

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MicroRNAs are small, noncoding RNAs that post-transcriptionally regulate gene expression. A polycistronic cluster of microRNAs, miR-17-92, is mis-expressed in a wide range of tumors and tumor cell lines. Ectopic expression of this microRNA cluster in cooperation with c-myc promotes development of B cell lymphoma in a mouse model. We hypothesize that inhibition of the microRNAs within this cluster is a therapeutic approach for the treatment of breast cancer. We undertook several strategies to test this using in vitro models. (1) Antisense inhibitors of microRNAs within the cluster exhibit cytotoxicity of carcinoma cells. This is dependent on nucleic acid modification chemistry. Locked nucleic acid modified antisense molecules exhibited the greatest potency but led to nonspecific toxicity. (2) We mapped the transcriptional start region and the transcriptional regulation of this microRNA cluster. This cluster is regulated by E2F family transcription factors. Interference with transcription is a possible therapeutic strategy. Targeting of the primary transcript is second novel therapeutic approach. We are currently testing these approaches.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0607 and National Institutes of Health.

P68-8: INHIBITION OF KLF6-SV1: INITIAL EXPLORATION OF A POTENTIALLY NOVEL BREAST CANCER TARGET

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The evolution possible through genetically defined breast cancer therapeutics is best exemplified by the increases in both overall and progression-free survival seen with targeted molecular therapies against HER2, which is amplified or overexpressed in a subset of cases. Further advances will similarly develop from identifying and targeting additional key genetic pathways underlying breast cancer progression and development. Recently overexpression of the oncogene KLF6-SV1, the alternative splice form of the tumor suppressor KLF6, was shown to be associated with an increased lifetime risk of prostate cancer while its targeted suppression led to decreased tumor cell proliferation, migration, invasion, and in vivo angiogenesis and tumorigenicity. Moreover, and suggestive of its role in a broad range of human cancers, KLF6 has been repeatedly identified among a handful of genes associated with worse clinical outcome, risk of recurrent disease, and marker of chemotherapeutic response in prostate, lung, and most recently, breast cancer.

During testing and characterization of highly serum-stable, high-efficiency siRNA targeting KLF6-SV1 in a number of cell lines, we made the serendipitous observation that KLF6-SV1 inhibition results in marked apoptosis in a number of breast cancer cell lines. To date, no studies have directly explored the functional role of KLF6-SV1 in breast cancer nor tested the therapeutic potential of KLF6-SV1 inhibition in vivo. Therefore, our hypothesis is that KLF6-SV1 inhibition represents a potentially novel breast cancer target.

Therefore, these initial studies explored the in vivo effectiveness of KLF6-SV1 inhibition on the clinically relevant tumor phenotype of breast cancer growth using a mouse-based, subcutaneous model. The breast cancer cell lines MDA-MB-435 (estrogen-independent) and MCF-7 (estrogen-dependent) were used. In vivo silencing of KLF6-SV1 was achieved by direct intratumoral injection of siSTABLE siRNA (Dharmacon; 10 mg/kg). In addition to effect on tumor growth, resultant tumor sections were analyzed for markers of cellular proliferation, apoptotic index, and angiogenesis.

Results will be discussed in context to effect on tumor growth and the novel pathways identified through these studies.

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P68-9: DEREGULATION OF DEATH-DOMAIN-ASSOCIATED PROTEIN IN BREAST CANCER CELLS

Xiaolu Yang, Jun Tang, Yide Mei, and Like Qu

University of Pennsylvania

The p53 pathway plays a critical role in preventing tumorigenesis through the induction of cell cycle arrest, apoptosis, or senescence in response to various stresses such as DNA damage. Defects in this pathway caused by mutations and dysfunctions in p53, its regulators and/or effectors lead to tumor formation. Mutations in p53 have been found in 50% of all human tumors, although only in 20% of breast tumors, suggesting that the disruption in p53 pathway in the majority of breast tumors is caused by dys-

functions of p53 regulators and/or effectors. We have recently revealed that the death-domain-associated protein (Daxx) is an important regulator in the p53 pathway. In unstressed cells, Daxx inhibits p53 function through binding and stabilizing the p53 E3 ubiquitin ligase, Mdm2. After DNA damage, Daxx dissociates from Mdm2, followed by accelerated Mdm2 degradation and p53 activation. Given the strong effects of Daxx on Mdm2 and p53, we hypothesize that Daxx may be deregulated in breast tumors and this deregulation may contribute to tumorigenesis. To test this, we first examined the expression of Daxx in tumors. We found that the Daxx locus is amplified and the Daxx protein is overexpressed in a large number of primary breast tumors and breast tumor cell lines. Immunohistochemical analysis further showed a correlation between Daxx overexpression and Mdm2 overexpression. To assess the role of Daxx in controlling the growth of tumors, we silenced Daxx in a breast cancer cell line, MCF7 cells. MCF7 cells treated with Daxx siRNA grew noticeably slower than the control cells. Our results suggest that Daxx may be overexpressed in breast tumors and contribute to tumor growth. In addition, we found that Daxx was rapidly phosphorylated in response to DNA damage in an ATM-dependent manner. We then mapped the potential phosphorylation sites by mutagenesis, kinase assay, and site-specific antibody. The data indicate that such phosphorylation may regulate p53 function. We will further examine the impact of the phosphorylation mutants of Daxx on tumorigenesis using in vitro systems and animal models.

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P68-10: ASSESSMENT OF GPR30, A SEVEN-TRANSMEMBRANE-SPANNING ESTROGEN RECEPTOR, AS AN ONCOGENE

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Expression of the seven-transmembrane-spanning receptor (7TMR), GPR30, has been linked to specific estrogen binding and rapid estrogen signaling resulting in stimulation of adenylyl cyclase and transactivation of the epidermal growth factor receptor, erbB1. More recent data has shown that expression of GPR30 in primary human breast tumors is positively associated with tumor progression variables including increased tumor size, HER-2/neu overexpression and the presence of extra mammary metastases (Filardo et al., 2006). Since altered expression and/or mutation of 7TMRs are associated with a wide spectrum of disease phenotypes, including cancer, this raises the possibility that GPR30 may function as an oncogene. To test this hypothesis, we have begun to generate transgenic mice that conditionally overexpress wild-type or mutant GPR30 under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter and determine the predisposition of these mice to develop invasive mammary carcinoma. We have produced two separate founder lines of MMTV-GPR30-WT mice. Breast tissue from nulliparous F1 progeny mice express low levels of GPR30 mRNA exhibit normal reproductive behavior and lactational competence yet no overt signs of cancer or premalignancy. Evidence of increased transgene expression and abnormal mammary gland growth or neoplasia is currently being monitored in multiparous mice. The results of our efforts regarding mutant GPR30 transgenic mice will be discussed.

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P68-11: ABERRANT EXPRESSION OF GERMLINE-SPECIFIC TRANSCRIPTION FACTOR OCT-4 AND MAMMARY TUMORIGENESIS

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Background and Objectives: The POU domain transcription factor Oct-4 (POU5F1 or Oct-3) is a member of the homeobox gene family and is specifically expressed in totipotent mouse and human embryonic stem (ES) and germ cells. It is considered to be a gatekeeper in the early steps of mammalian embryogenesis because of its pivotal role in the regulation and maintenance of pluripotency (a widely used marker of pluripotency) and self-renewal of ES cells. Expression of Oct-4 is downregulated when embryonic stem cells are triggered to differentiate and is lost in normal somatic cells of differentiated tissues. However, there is increasing evidence showing that Oct-4 regains its expression in some cancer cells, possibly linking Oct-4 to carcinogenesis. Reactivation of Oct-3/4 expression is observed in several human breast cancer cell lines and in human primary breast carcinomas but not in normal human breast tissue. We hypothesize that aberrant Oct-3/4 expression contributes to the neoplastic process in mammary gland tumorigenesis and/or to the maintenance of the transformed state of cancer cells. The specific aim of our Seed Grant proposal was to generate conditional transgenic mice that express human Oct-4 placed under the regulatory control of the tet operator promoter (tetO) and a tetracycline-dependent transactivator protein (rtTA) under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR).

Methodologies: We first cloned human Oct-4 cDNA into a Tet-on vector pBI-EGFP-Tet (Clontech) and obtained the MMTV-rtTA plasmid from Dr. L.A. Chodosh. The DNA fragments of hOct-4-pBI-EGFP-Tet DNA and the MMTV-rtTA were excised from these plasmids and injected into fertilized oocytes to produce double transgenic mice.

Results: Transgenic mice (C57BL/6) were screened for MMTV, rt-TA, EGFP, Tet, and pBI sequences in their genome by RT-PCR and Slot blot analyses. Four founder lines have been identified to carry the double transgenes. Figure 1 shows the immunofluorescent staining of a transgenic F1 female virgin mouse fed doxycycline for 7 days, which shows strong inducible expression of both hOct-4 and EGFP.

Conclusions: We have successfully generated double transgenic mice with temporal and spatial Oct-3/4 expression in the mammary gland using a MMTV-LTR controlled, tetracycline responsive system. These conditional transgenic mice will provide a very useful model to study the roles of Oct-4 in mammary cancer initiation and progression.

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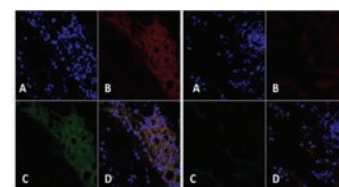


Fig. 1. Immunofluorescence analysis of Oct-4 expression in the mammary gland of Oct-4 transgenic mice with (left panels) or without (right) doxycycline induction. A. DAPI staining; B. Oct-4 staining; C. EGFP fluorescence; D. Oct-4 and DAPI staining.

P68-12: Akt1—A NEW TARGET FOR HORMONE THERAPY?

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Introduction: We have previously demonstrated that Akt1 is activated not only by epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), and heregulin- β 1 (HRG- β 1), but also by estradiol. This cross-talk between growth factors and estrogen receptor- α (ER- α) signaling converges in the EGF receptor family member, ErbB2 / phosphatidylinositol 3-kinase PI 3-K/serine/threonine protein kinase Akt pathway in hormone-dependent MCF-7 breast cancer cells in vitro and xenografts grown in vivo. The aim of the present study was to investigate whether the current model of this tumorigenic pathway is active in clinical breast cancer.

Methods: We analyzed the expression levels of Akt1 and phosphorylated Akt (pAkt) by immunohistochemistry and western blot analysis in pairs of frozen tumor samples and their corresponding matched normal adjacent lobular breast tissue from 58 breast cancer patients. These patients did not have a previous history of therapeutic intervention. The tumors were mostly invasive, high-grade, ductal carcinomas, with an approximately equal distribution of ER- α , ErbB2, and lymph node positivity. Spearman's rank correlations were calculated for Akt expression and phosphorylation, measured by either immunohistochemistry or western blot, and patient and clinical-pathological tumor classification.

Results: Marked staining was observed for Akt1 in 98% of the tumors and pAkt was expressed in 83%. Akt1 was localized mostly in the cytoplasm, but also in the nucleus. Normal tissues revealed much less staining for Akt1 and pAkt than tumor tissue, also with more cytoplasmic localization and less nuclear staining. Nuclear Akt1 measured by immunohistochemistry was highly correlated with Akt1 expression measured by western blot analysis (Akt-WB) ($p=0.04$), cytoplasmic Akt1 (cyt Akt) ($p=0.006$), and nuclear pAkt ($p=0.003$). Additionally, nuclear pAkt was highly correlated with phosphorylated cytoplasmic Akt (cyt pAkt) ($p=0.001$). Akt1 and pAkt expressions were not significantly correlated with age, tumor type, nuclear grade, lymph node positivity, progesterone receptor (PR), or the proliferation rate, Ki67. Nuclear Akt1 and cyt pAkt were each significantly correlated with tumor size ($p=0.02$). Cyt pAkt was positively correlated with histological grade ($p=0.02$) and inversely correlated with ER- α status ($p=0.04$) and with ErbB2 expression ($p=0.05$). Nuclear pAkt was also inversely correlated with ErbB2 ($p=0.04$). pAkt measured by western blot (pAkt-WB) was also associated with tumor size ($p=0.03$).

Conclusion: Similar to our in vitro model and animal data, in invasive, high-grade, ductal carcinomas, Akt expression and activity are high and correlate with ErbB2 and ER- α expression, histological grade, and tumor size.

Keywords: Akt1, ErbB2, ER- α , histological grade, tumor size

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0684.

P68-13: RNAi AS A TOOL FOR DETERMINING THE ROLE OF CARCINOEMBRYONIC ANTIGEN IN BREAST CANCER

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Carcinoembryonic antigen (CEA) is overexpressed in a variety of cancers, including cancer of the breast. Recent studies suggest that deregulated overexpression of CEA promotes tumorigenesis by inhibiting cell differentiation of many cell types and by preventing anoikis, a type of apoptotic program that destroys cells that lose contact with the extra cellular matrix. Significantly, downregulation of CEA in human HT29 colon cancer cells has been shown to inhibit tumor cell aggregation and the reduction in colony formation. Although these findings indicate that CEA might have a role in tumor progression, if and how CEA promotes breast cancer is not known. We hypothesized that if deregulated overexpression of CEA is linked to breast cancer progression, downregulation of CEA expression via destruction of its mRNA may promote apoptosis or alter the expression of genes that are involved in apoptosis and cell cycle/proliferation. To investigate the relationship between overexpression of CEA and breast cancer progression, we employed two chemically synthesized siRNAs (siRNA 497 and 2043) to downregulate CEA mRNA. To monitor the cellular uptake, nonsilencing control siRNA was labeled with fluoresceine at the 5' end of the antisense strand. The siRNA transfected MCF7 cells were incubated at 37°C for 24, 48, and 72 h, and cell lysates were resolved on a 4-20% SDS-polyacrylamide gradient gel, transferred to polyvinylidene fluoride (PVDF) membrane and probed with anti-CEA antibody. Unlike nonsilencing control, both of the siRNAs displayed significant downregulation of CEA protein with siRNA 497 more effective than siRNA 2043. Our data indicate that treatment of MCF7 cells with siRNA 497 siRNA arrested cell cycle at G1/S transition after 48 h as compared to scrambled siRNA.

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P68-14: IDENTIFICATION OF ErbB-2 KINASE DOMAIN MOTIFS REQUIRED FOR GELDANAMYCIN-INDUCED DEGRADATION

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The ansamycin antibiotic geldanamycin (GA) induces the intracellular degradation of ErbB-2/neu. Degradation of ErbB-2 proceeds through cleavage(s) within the kinase domain, resulting in the formation of a 135 kDa ectodomain fragment and a fragment(s) of 50 kDa containing the COOH-terminal region. On the basis of independent means of identification, two adjacent sequence motifs have been identified in ErbB-2 that are required for GA-induced degradation. These motifs encompass residues 776-783 and 784-786 within the NH₂-terminal lobe of the ErbB-2 kinase domain. This is also a region in which the epidermal growth factor receptor and ErbB-2 kinase domains differ significantly in sequence. Although mutations in this region abrogate GA-induced ErbB-2 degradation, the tyrosine kinase activity of ErbB-2 is not disrupted. Interestingly, these ErbB-2 mutants are specifically resistant to GA-induced degradation but retain sensitivity to other drugs, such as staurosporine and curcumin, which are also able to provoke ErbB-2 degradation.

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P68-15: EGFR COOPERATES WITH STAT3 TO INDUCE EPITHELIAL-MESENCHYMAL TRANSITION IN CANCER CELLS VIA UPREGULATION OF TWIST GENE EXPRESSION

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Aberrant EGFR signaling is a major cause of tumor progression and metastasis; the underlying mechanisms, however, are not well understood. In particular, it remains elusive whether deregulated EGFR pathway is involved in epithelial-mesenchymal transition (EMT), an early event that occurs during metastasis of cancers of an epithelial origin. Here we show that EGF induces EGFR-expressing cancer cells to undergo a transition from the epithelial to the spindle-like mesenchymal morphology. EGF reduced E-cadherin expression and increased that of mesenchymal proteins. In search of a downstream mediator that may account for EGF-induced EMT, we focused on transcription repressors of E-cadherin, TWIST, SLUG, and Snail and found that cancer cells express high levels of TWIST and that EGF enhances its expression. EGF significantly increases TWIST transcripts and protein in EGFR-expressing lines. Forced expression of EGFR re-activates TWIST expression in EGFR-null cells. TWIST expression is suppressed by EGFR and Jak/STAT3 inhibitors but not significantly by those targeting PI3K and MEK. Furthermore, constitutively active STAT3 significantly

activates the TWIST promoter whereas the JAK/STAT3 inhibitor and dominant-negative STAT3 suppressed TWIST promoter. Deletion/mutation studies further show that a 26 bp promoter region contains putative STAT3 elements required for the EGF responsiveness of the TWIST promoter. Chromatin immunoprecipitation assays further demonstrate that EGF induces binding of nuclear STAT3 to the TWIST promoter. Immunohistochemical analysis of 130 primary breast carcinomas indicates positive correlations between non-nuclear EGFR and TWIST and between p-STAT3 and TWIST. Together, we report here that EGF/EGFR signaling pathways induce cancer cell EMT via STAT3-mediated TWIST gene expression.

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P68-16: ATM-MEDIATED PHOSPHORYLATION OF DEATH-DOMAIN-ASSOCIATED PROTEIN REGULATES DNA DAMAGE-INDUCED p53 ACTIVATION

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Disruption of the p53 tumor suppression pathway contributes to nearly all human tumors. However, only 20% of breast cancers harbor mutations in p53, suggesting that the remaining 80% of breast cancers are associated with mutations/dysfunction in p53 regulators and/or effectors. We have recently shown that the death-domain-associated protein (Daxx) plays a critical role in suppressing p53 function in unstressed cells by binding and stabilizing E3 ubiquitin ligase Mdm2 through the ubiquitin-specific protease Hausp. Upon DNA damage, Daxx disassociates from Mdm2 prior to Mdm2 degradation and p53 activation. We have also shown that endogenous Daxx and p53 associate with each other. In addition, our preliminary data suggest that Daxx may be aberrantly overexpressed in a substantial number of breast cancer cells and tissues. These results implicate Daxx as an important regulator in the p53 system, and that dysregulation of Daxx may contribute to tumorigenesis. To assess the role of Daxx in p53 activation and regulation of the Daxx-Mdm2 complex after DNA damage, we examined DNA damage-induced Daxx phosphorylation. We found Daxx is rapidly phosphorylated upon DNA damage, and this phosphorylation is mediated by ataxia-telangiectasia mutated (ATM) kinase both in vitro and in vivo. We then mapped the major phosphorylation site on Daxx and confirmed its phosphorylation by using a site-specific phospho-antibody. Interestingly, this phosphorylation site does not appear to regulate Mdm2/Daxx interaction, but instead is critical for Daxx binding to p53. Our results suggest that ATM-mediated Daxx phosphorylation may play an important role in DNA damage-induced p53 activation.

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P68-17: VULNERABILITY OF NORMAL HUMAN MAMMARY EPITHELIAL CELLS TO ONCOGENIC TRANSFORMATION

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Recent studies have shown that cells derived from breast tumors display great diversity in patterns of gene and protein expression and genomic alterations. Based on gene expression profiling, multiple distinct cancer subtypes have been categorized; importantly, these subtypes show striking differences in clinical parameters. Understanding the pathways of molecular alterations that lead to the different types of breast cancer in vivo could facilitate design of clinical interventions in the carcinogenic progression. One approach to examining human breast carcinogenesis is to model this process in vitro, starting with normal HMEC and using oncogenic agents to transform the normal cells to cancer. However, thus far, almost all in vitro transformed HMEC lines represent a limited subset of the phenotypes observed in breast cancer cells in vivo. We hypothesized that the limited phenotypes of in vitro transformed HMEC could result from culture conditions that restrict proliferation of most normal HMEC. Normal cultured HMEC proliferate for a variable number of population doublings (PD) before encountering a first senescence barrier, stasis, which is stress-associated, mediated by the retinoblastoma (Rb) pathway, correlated with increased levels of p16INK4a, and telomere length independent. HMEC may overcome stasis by inactivation of the Rb pathway and continue growth until encountering a second extremely stringent barrier due to telomere attrition. HMEC grown in a serum-free medium reach stasis quickly and can show spontaneous silencing of p16, giving rise to the p16(-) post-stasis HMEC (called post-selection) that are commercially available. In vitro transformed lines derived from post-selection HMEC have shown a basal phenotype. We now propose to generate HMEC lines more reflective of the in vivo spectrum of breast cancer phenotypes by using improved methods for growing normal pre-stasis HMEC. We have defined low stress culture conditions that allow pre-stasis HMEC to grow for ~60 PD prior to p16 induction and growth arrest at stasis. Pre-stasis HMEC with phenotypes of luminal, basal, and progenitor lineages can be identified, supporting our belief that these cells in these cultures more accurately represent in vivo populations. We hypothesize that these heterogeneous unstressed pre-stasis populations will be more

vulnerable to transformation when targeted by oncogenic agents and may yield cell lines with a greater range of transformed phenotypes. Our objectives include addressing the basic research questions—are unstressed pre-stasis HMEC more vulnerable to transformation and is a particular normal cell type more vulnerable or more likely to give a specific transformed phenotype, and producing a practical outcome - generation of useful transformed lines. Our approach will be: (1) Determine whether transduction of unstressed pre-stasis HMEC with oncogenes such as c-myc and Wnt-1, with or without p53 inactivation, will yield transformed lines with phenotypes representative of most human breast cancer cells and if there are correlations of transformed cell phenotypes with the target cell population and/or agents used for in vitro transformation. (2) Experimentally examine whether cultured HMEC that have not encountered stress are more vulnerable to transformation than those exposed to stress. The generation of diverse transformed HMEC lines with defined genetic alterations may aid the identification of potential therapeutic treatments.

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P68-18: TUMOR CHAPERONE PROTEIN SYNUCLEIN GAMMA (SNCG) CHAPERONES ER AND PI3K-Akt-mTOR SIGNALING PATHWAY AND RENDERS ENDOCRINE AND DRUG RESISTANCE

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Background: We previously identified a breast cancer specific gene, BCSG, also named as SNCG. Expression of SNCG is strongly correlated with the stage, lymph node involvement, and metastasis. Patients with an SNCG-positive breast cancer have a significantly shorter disease-free survival and a high probability of death compared with patients with no SNCG expression.

Data: SNCG participates in the heat shock protein (Hsp)-based multiprotein chaperone complex and regulates many pathways in growth and progression of cancer, including (1) stimulation of ER α transcriptional activation and hormone-dependent growth and tumorigenesis and (2) chaperoning and stimulating PTEN/Akt/mTOR signaling pathway. SNCG physically interacts and transactivates HER2 and activates its downstream Akt pathway in breast cancer cells. While treatment of SNCG-negative breast cancer cells with Hsp90 inhibitor 17-AAG significantly inhibits PI3K and mTOR activation, expression of SNCG abolishes this 17-AAG-mediated inhibition of PI3K and mTOR activation. Expression of SNCG also significantly increases endocrine- and drug resistance.

Significance: Clinical follow-up studies indicate that expression of SNCG in breast cancers renders resistances to adjuvant therapy. Our study suggests that SNCG, as a tumor-specific chaperone, maintains PI3K/Akt/mTOR signaling pathway integrity and thus renders tumor cells to drug resistance.

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STROMAL- EPITHELIAL INTERACTIONS II

Poster Session P69

P69-1: MODULATION OF NF- κ B IN THE MACROPHAGE LINEAGE: EFFECTS ON MAMMARY DEVELOPMENT AND TUMORIGENESIS

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Morphogenesis of the mammary gland is a highly complex process that when misregulated can result in tumorigenesis. It involves the interactions of multiple cell types in a highly regulated manner with complex signal transduction pathways coordinating the physiological processes. Interactions between epithelial and mesenchymal cells are known to be important. Recent studies highlight the importance of cell types, such as macrophages. The nuclear factor-kappa B (NF- κ B) family of transcription factors appears to be critical in regulating the dynamic changes during normal and neoplastic development. Our study investigates the contribution of NF- κ B signaling within macrophages in normal and neoplastic mammary development. We have used two approaches to address this question. In the first, we have generated macrophage cell lines from mice in which the major inhibitor of NF- κ B (I κ B- α) has been deleted. These cells have constitutive NF- κ B activation. We have identified differences in proliferation rates, expression of downstream target genes such as MMP9, and effects mediated by altered macrophages on associated epithelial cells. Our second approach has been the development of an inducible transgenic system that allows us to modulate NF- κ B activity in macrophages in vivo. In this system, the expression of the reverse tetracycline transactivator (rtTA) is targeted to macrophages using the c-fms promoter. These transgenics are crossed with mice that express either a constitutive activator (cI κ B) or a dominant inhibitor (I κ B α -DN) under the regulation of an rtTA responsive promoter. Treatment of double transgenic mice with doxycycline in drinking water turns on the expression of the relevant inducible transgene. Using this system, we are able to both up- and downregulate NF- κ B activity in specific temporal and developmental windows in cells of the macrophage lineage in vivo. Our data suggest that altered NF- κ B activity within macrophages has significant effects on virgin mammary ductal development. Our studies may identify NF- κ B signaling within macrophages as an important contributor to mammary tumorigenesis and thus as a potential therapeutic target.

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P69-2: ROLE OF MAMMARY GLAND INVOLUTION IN PROMOTING METASTASIS IN PREGNANCY-ASSOCIATED BREAST CANCER (PABC)

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Full-term pregnancy increases breast cancer risk for up to 10 years after pregnancy and breast cancer diagnosed during this time has been referred to as PABC (Albrektsson, *Br. J. Cancer*, 2005). Given this definition, an estimated 30,000 U.S. breast cancer cases a year could be complicated by prior pregnancy. Importantly, PABC patients have high rates of metastasis, which is independent of known prognostic factors. Following parturition or lactation, during involution, the breast returns to its pre-pregnant state. In a rat model, we found mammary gland involution employs tissue remodeling programs activated during wound healing and inflammation including high matrix metalloproteinase -2, -3, and -9 activities, release of bioactive fragments of FN and LN, deposition of fibrillar collagen, and increased cytokine levels, including TGF β 1, TGF β 3, and connective tissue growth factor (McDaniel, *AJP*, 2006, & Schedin, *JMB&N*, 2007). We hypothesize that this physiologically normal, but pro-inflammatory remodeling of the gland accounts for the high rate of metastases seen in PABC (Schedin, *Nature Reviews Cancer*, 2007).

To further define involution features common to inflammation, the question of whether macrophages are recruited to the involuting gland was investigated in both rat mammary and human breast tissue. Macrophages have been shown to be obligate partners for mammary tumor cell metastases in animal models and increased macrophage density in human breast cancer correlates with poor prognosis. To determine whether macrophage number is increased during involution, mammary tissue from virgin, pregnant, lactating, involuting and fully regressed mammary tissue was stained for macrophage marker CD68. CD68 is a lysosomal-associated protein. In rat mammary glands, the number of CD68 positive cells increased more than 6-fold during involution. Involution-macrophages were identified as subtype M2-like by arginase-1 expression. These macrophages did not express the subtype M1-marker iNOS. Tumor-associated macrophages (TAM) are subtype M2-like, whereas tumor suppressor macrophages are M1-like. These data are consistent with involution being characterized by a pro-tumorigenic microenvironment.

The question of whether breast involution in women also is characterized by a pro-tumorigenic microenvironment is unanswered. To address this question, formalin-fixed breast biopsy tissues were obtained for women who were either pregnant (N=3), lactating (N=7) or whose breast tissue was actively involuting (N=4) at the time of biopsy. CD68 staining in human breast tissue followed the same trend, with 5 times more

staining in involuting compared to pregnant or lactating tissue. Total leukocyte number, as assessed by CD45 antibody, and the oncofetal ECM protein tenascin were also increased in involuting human breast tissue. With respect to these three stromal markers, involuting breast tissue was more similar to tumor stroma (N=5) than stroma from breasts of nulliparous women (N=3). These data demonstrate for the first time that mammary gland involution in women is characterized by a pro-inflammatory microenvironment, implicating physiologic tissue inflammation in the poor prognosis of PABC.

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P69-3: 3D ORGANOTYPIC MODELS OF DUCTAL CARCINOMA IN SITU (DCIS): MODELS FOR ASSESSING STROMAL EFFECTS ON PROGRESSION TO INVASION AND INCREASED PROTEOLYSIS

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Most breast cancers progress from morphologically distinct pre-invasive lesions (DCIS) to invasive carcinomas. Often, sites of transition from DCIS to invasive carcinoma are characterized by a breakdown of the basement membrane and an increased number of stromal cells and cytokines. We have sought to mimic in vitro the in vivo tumor microenvironment through organotypic co-cultures in which comedo-type DCIS cells derived from MCF-10A human breast epithelial cells (MCF10.DCIS.com) are grown in a three dimensional (3D) co-culture of reconstituted basement membrane (rBM) with either normal human breast fibroblasts or human breast myoepithelial cells. We also analyzed the effects of co-culture with HGF over-secreting fibroblasts in order to mimic the elevated levels of HGF often found within the activated breast tumor microenvironment. Along with these contact co-cultures, conditioned media from tumor-stimulated stromal cultures was used to evaluate effects on DCIS morphology, growth, progression to invasion, and proteases thought to be involved in ECM turnover. Live images of co-cultures revealed that both fibroblast lines migrated actively, whereas DCIS cells formed stationary disorganized spheroids that eventually connected with neighboring structures. In co-culture with HGF-secreting fibroblasts, DCIS cells formed dynamic structures that developed invasive foci and degraded the rBM. By adding myoepithelial cells, we can reciprocate in vivo morphology in which the myoepithelial cells form a single layer surrounding luminal epithelial cells. Myoepithelial co-culture with DCIS cells reversed or delayed DCIS progression by inhibiting disorganized growth and the onset of invasive foci. A novel confocal microscope based proteolysis assay was used to show that conditioned media of fibroblasts and myoepithelial cells increased and decreased, respectively, the degradation of both type I and type IV collagen by DCIS cells. Employing cysteine cathepsin selective activity-based probes, we are able to co-localize cysteine cathepsin activity with intracellular cleavage products of type IV and type I collagen. We have created several in vitro 3D organotypic models that mimic the changing microenvironments (myoepithelial contact, normal fibroblasts and activated fibroblasts) of DCIS in vivo. We are in the process of analyzing the proteases and protease inhibitors in the 3D organotypic DCIS co-cultures.

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P69-4: A ZEBRAFISH GENETIC MODEL OF THE BREAST CANCER MICROENVIRONMENT

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Despite improved treatments, metastatic breast cancer kills more than 40,000 women each year in the United States. Analysis of metastatic breast cancer cell lines has revealed many factors produced by the tumor cells that promote metastasis, for example matrix metalloproteinases, adhesion molecules and angiogenic growth factors. In contrast, relatively little is known about what factors in the host contribute to the establishment of metastases. It is critically important to discover other such host factors; however, the tumor microenvironment has been difficult to study because it cannot be manipulated as easily as the cell lines. To address this problem we have turned to the zebrafish, a powerful, genetically tractable vertebrate model system with cancer biology very similar to human. Recently, it was shown that human melanoma cell xenografts survive and stimulate angiogenesis when placed in zebrafish embryos (Haldi et al., *Angiogenesis* 2006). We will introduce fluorescently labeled breast cancer cells into the transparent embryos and assess the survival, growth and spread of the cells. To identify critical host factors, we will use knockdown and mutagenesis techniques to systematically alter the tumor microenvironment, assessing the impact on growth and invasiveness of the xenografted cells. The goal of this study is to understand how the host microenvironment influences the development of breast cancer metastasis.

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P69-5: CD151 PROMOTES BREAST TUMOR PROGRESSION BY REGULATING $\alpha 6$ INTEGRIN FUNCTIONS, SIGNALING, AND MOLECULAR ORGANIZATION

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Background and Objectives: There is ample evidence that expression of integrin $\alpha 6 \beta 4$ is associated with the estrogen receptor-negative basal-like subtype, high tumor grade, and increased mortality. Also, $\alpha 6 \beta 4$ appears to promote mammary tumorigenesis by amplifying signaling of ErbB family members. CD151, a master regulator of laminin-binding integrins ($\alpha 6 \beta 4$, $\alpha 6 \beta 1$, and $\alpha 3 \beta 1$), assembles these integrins into complexes called tetraspanin enriched microdomains (TEMs). At present, little has been done regarding CD151 in breast cancer. Given CD151 association with laminin-binding integrins, we hypothesized that CD151 influences mammary tumor progression. The goal of our studies was to explore the role of CD151 during human breast tumor malignancy and to determine whether it affects breast tumor progression in an integrin-dependent manner.

Brief Description of Methodologies: To achieve our goals (1) we carried out immunohistochemical staining of CD151 proteins on human breast cancer tissue array samples, (2) both RNAi and stable shRNA approaches were taken to ablate CD151 in representative human mammary cell lines to assess its effects on cell biology in vitro, and (3) we analyzed tumor-forming properties of CD151-ablated malignant MDA-MB-231 cells in vivo by using ectopic and orthotopic xenograft models of human breast cancer.

Results to Date: Initially, by staining annotated human breast tumor arrays with CD151-specific monoclonal antibody, we found elevated CD151 in high-grade and ER-negative tumors, including the triple negative basal-type human breast cancers. To gain molecular insights into CD151 action, we ablated this protein in two representative human mammary cell lines (normal MCF-10A and malignant MDA-MB-231) via RNA and stable shRNA. In both of these lines, CD151 ablation yielded marked alterations in integrin-mediated cell migration, invasion, spreading, and signaling (through FAK, Rac1, and Ick) while disrupting EGFR- $\alpha 6 \beta 4$ integrin collaboration. Underlying these defects, CD151 ablation redistributed $\alpha 6 \beta 4$ integrins subcellularly and severed molecular links between integrins and TEMs. In a prototypical basal-like mammary tumor line (MDA-MB-231), CD151 ablation notably delayed tumor progression in ectopic and orthotopic xenograft models.

Conclusions: These results (1) establish that CD151- $\alpha 6 \beta 4$ complexes play a functional role in basal-like mammary tumor progression, (2) emphasize that $\alpha 6$ integrins function, via CD151 linkage, in the context of TEMs, and (3) point to potential relevance of CD151 as a high priority therapeutic target with relative selectivity (compared to laminin-binding integrins) for pathologic rather than normal physiology.

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P69-6: PASSAGE OF MICRORNA FROM BONE MARROW STROMA TO BREAST CANCER CELLS VIA GAP JUNCTIONS: CONSEQUENCE FOR CANCER CELL QUIESCENCE CLOSE TO THE ENDOSTEUM

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Previous failure of autologous bone marrow transplantation as a viable treatment of breast cancer opens the field for investigational studies since the bone marrow, as a source of cancer metastasis, remains a clinical dilemma. This study hypothesizes that a subset of breast cancer cells enter the bone marrow at an early period of disease development, perhaps prior to clinical detection, where they evade chemotherapeutic damage even at high doses. An understanding of this cell population might be crucial for the future success of bone marrow transplants in patients suffering from breast cancer metastasis to the bone marrow. In vivo and in vitro studies have demonstrated functional gap junctions between bone marrow stroma and breast cancer cells. We have now implemented functional studies to determine if the gap junctions could be a mechanism by which the stromal cells could influence cancer cell quiescence. This question was studied by determining if specific microRNAs to the oncogenic *Tac1* mRNA (miRNAs 130a and 206) could pass from stroma to breast cancer cells (T47D and MDA-MB-231), and to prevent its translation. By qPCR, transfection with anti-miRs and reporter gene assays, we have determined synergism between the two miRNAs in the suppression of *Tac1* expression. Finally, we determined whether the passage of miRNA from stroma to breast cancer represents a global mechanism in bone marrow. This was addressed by analyzing the miRNAs in T47D and stromal cells, before and after co-cultures. We observed similar levels of miRNA in stroma and breast cancer cells after gap junction development, suggesting a mechanism by which

cancer cells adapt a quiescence phenotype, and to evade detection and treatment. Further understanding of these mechanisms might lead to new methods to eradicate breast cancer cells from bone marrow by the development of new cancer therapies.

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P69-7: INFLUENCE OF c-KIT EXPRESSION IN STROMA ON BREAST CANCER GROWTH AND RESPONSE TO TARGETED THERAPY

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Previous studies have shown that there is a progressive loss of c-Kit expression in breast epithelial tissue as this tissue changes from normal to benign to cancerous while simultaneously there is a gain in c-Kit expression in the surrounding stromal tissue. We have studied this inversion in c-Kit expression in an organotypic environment in vivo and in culture to determine its influence on tumor growth and tumor chemosensitivity. Human breast cancer cells (Hs578T) and autologous surrounding fibroblasts (Hs578Bst) were admixed and grown as xenografts in the mammary fat pad of immune-deficient mice and in culture on a layer of collagen I in Mat Tek dishes. Overexpression of c-Kit in fibroblasts was carried out using Lipofectamine-mediated transfection of pcDNA3/c-Kit. Chemosensitivity to the c-Kit modulating drugs, imatinib and alpha-fetoprotein-derived peptide (AFPeP), was evaluated. Xenografted tumor cells grew only when injected in admixture with fibroblasts. Growth was accentuated when fibroblasts overexpressed c-Kit. Both imatinib and AFPeP inhibited tumor growth when tumor was inoculated with its normal fibroblasts. However, within the context of c-Kit-overexpressing fibroblasts, tumor lost sensitivity to growth inhibition by imatinib but not to AFPeP. In culture, tumor became more rounded when grown in the context of fibroblasts. In the presence of imatinib, tumor growth and migration were inhibited, and fibroblasts were induced into a dendritic morphology. Overexpression of c-Kit in fibroblasts prevented the imatinib-induced changes in morphology and migration. In the presence of AFPeP, tumor growth was inhibited, but there were no changes in tumor migration or fibroblast morphology, and overexpression of c-Kit in fibroblasts did not significantly impact response of tumor to AFPeP. The results of this study indicate that the presence of stroma and particularly c-Kit expression in stroma can influence the growth rate of breast cancer as well as the response of that cancer to targeted therapy.

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P69-8: SECOND HARMONIC GENERATION IMAGING OF STROMAL CHANGES DURING MOUSE MAMMARY TUMOR DEVELOPMENT

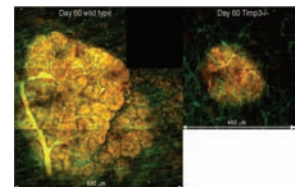
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Introduction: Second harmonic generation (SHG) allows the imaging of fibrillar collagen in tissues in its native state without the need for exogenous staining or fixation. It is a powerful tool to monitor changes in breast cancer stroma, which acts as a critical mediator of tumor progression. We know that mice lacking an important stromal mediator, the tissue inhibitor of metalloproteinase *timp3*, have delayed mammary tumor growth driven by the MMTV-PyMT transgene. We hypothesize that SHG and multiphoton imaging will provide insights into *timp3* effects on the stromal compartment of developing mammary tumors.

Methods: To determine the effect of fibrillar collagen content on SHG signal intensity, we performed multiphoton microscopy and transmission electron microscopy (TEM) on the same tissue and quantified the collagen fibril density and its relationship to SHG signal. Mammary glands were then isolated from MMTV-PyMT and MMTV-PyMT/*timp3*^{-/-} mice at defined stages of tumorigenesis (early lesions, intermediate, and established tumors). These mammary glands were examined ex vivo using multiphoton microscopy to visualize SHG from fibrillar collagen and cellular autofluorescence from the developing tumor mass.

Results: Quantitative image analysis of TEM and SHG microscopy on the same tissue (the liver) revealed that SHG emission intensity is proportional to the number of collagen fibrils per fiber with the exception of small fibers containing <300 fibrils or <1.5 micron diameter. This helps define the population of collagen fibers being sampled with SHG imaging and reveals the relationship between SHG signal and the molecular ultrastructure of collagen fibers within tissues. Using mammary gland explants, we



SHG image of WT and Timp3-/- mammary tumors

performed quantitative analysis on structural and morphological differences between mammary tumors developing in WT and *timp3*^{-/-} mice. We found the collagen fibers at the tumor periphery moved from a network of highly convoluted bent fibers to a more linear configuration with tumor growth, likely as a result of the underlying tumor distorting the collagen network. Comparing the same time point of tumor growth, we observed that WT tumors were generally larger than their *timp3* counterparts. As seen in the figure (SHG as green, autofluorescence as red), both sets of developing tumors were vascularized; however, WT tumors contained larger and more frequent blood vessels. Quantitative image analysis revealed that WT tumors were surrounded by a less dense collagen network than *timp3*^{-/-} tumors. We are currently following up by determining the source of these differences and their relation to *timp3* loss within the mammary gland.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0624.

P69-9: THE EFFECT OF THREE-DIMENSIONAL GROWTH ON EPITHELIAL-TO-MESENCHYMAL TRANSITION IN HUMAN BREAST CANCER CELLS

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The breast cancer microenvironment poses a formidable barrier to effective therapy. The pathophysiologic mechanisms underlying treatment resistance in breast cancer are not known; in part, this is due to the lack of a suitable, well-characterized model. This study's intent was to develop a three-dimensional breast cancer culture system capable of reproducing the tumor microenvironment. We cultured MCF-7, a human breast cancer cell line, under three conditions: on polystyrene, on a two-dimensional Collagen Type 1 gel, and on a three-dimensional (3D) Collagen Type 1 gel. The extent of phenotypic abnormality was assessed by changes in morphology on phase-contrast images and alterations in gene expression by qRT-PCR. G3PDH was used as an endogenous control, and relative quantification was performed using MCF-7 cells grown on polystyrene alone as the calibrator or reference sample. Genes tested included E-cadherin, c-kit, TGF- β , MMP-2, MMP-12 and MMP-19, chosen for their importance in cell-matrix signaling. Expression of E-cadherin, c-kit, MMP-2, MMP-12, and MMP-19 in MCF-7 grown on polystyrene was significantly higher than their expression in MCF-7 grown in 3D. TGF- β expression was significantly higher in MCF-7 cells grown in 3D compared to polystyrene. Gene expression in the two-dimensional gels trended toward that seen in the three-dimensional gels but was not statistically significant. MCF-7 cells grown in 3D formed small, loose aggregates in comparison to the stellate appearance of cells grown on polystyrene. In conclusion, we can nondestructively grow and image live breast cancer cells using optical methods. We were able to build an improved, precisely defined synthetic scaffold that better replicates the gene expression changes seen in human breast cancer, demonstrate that 3D growth affects the expression of genes important in cell-cell adhesion and cell invasion, and nondestructively image these cells. We hope this will lead to better understanding and targeted treatment in patients with treatment-resistant breast cancer.

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P69-10: TUMOR-DERIVED Cyr61(CCN1) PROMOTES STROMAL MATRIX METALLOPROTEINASE PRODUCTION AND ANGIOGENESIS

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Introduction: The highly complex mechanism of cancer cell-stromal cell MMP-induction and regulation during angiogenesis is not well understood, and the identification of the factors that mediate this complex process may enable the development of novel therapeutics. We hypothesized that tumor-derived Cyr61 can stimulate MMP production from adjacent stromal cells that may contribute to invasion and angiogenesis.

Methods: To examine a potential paracrine role of Cyr61, we have set up an in vitro co-culturing model system developed in our laboratory. Fibroblasts and/or endothelial cells were used as stromal cells in this system. Three different carcinoma cell lines were examined for their ability to induce MMP-1. Real-time PCR and western blot analysis were used to evaluate Cyr61 expression levels and MMP-1 levels upon co-culturing.

Results: The presence of MDA-MB-231 cells caused a 3.5-fold induction of MMP-1 mRNA in W138 stromal fibroblasts after 24 hours of co-culture as compared to the control W138 cells grown in RPMI alone. Moreover, 10 nM 17- α estradiol stimulation of MCF-7 cells induced W138 cells to express MMP-1 mRNA. Together, these co-culturing data show that expression levels of Cyr61 in the breast cancer cell correlate with induction of fibroblast MMP-1. Similar experiments are being carried out with endothelial cells. To demonstrate that cancer cells mediate their MMP1-inducing effect

via Cyr61, we silenced Cyr61 gene expression in breast cancer cells with siRNA. Treatment of MDA-MB-231 or MCF7-N55 cells with Cyr61 siRNA caused a major loss (71%) of MMP-1 induction from W138 fibroblasts as compared to control cells treated with luciferase siRNA.

Conclusions: These results provide the first direct evidence that tumor cell-secreted angiogenesis factor Cyr61 is a major paracrine regulator of stromal MMP-1. These results are currently being validated in vivo systems.

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P69-11: ROLE OF CELL ADHESION MICROENVIRONMENT AND THE Src-Stat3 AXIS IN AUTOCRINE HGF SIGNALING DURING BREAST TUMORIGENESIS

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Overexpression of both hepatocyte growth factor (HGF) and its receptor Met frequently occurs in invasive human breast cancer, suggesting that the establishment of an HGF "autocrine loop" may be linked to breast tumor progression. We have recently shown a novel activating function of two signaling molecules, Src tyrosine kinase and the signal transducer and activator of transcription-3 factor (Stat3), on HGF expression in breast epithelial cells. This activation was dependent on a Stat3 recognition element present at nt-95 in the HGF promoter (*Mol. Cancer*, 6:69-86, 2007). Interestingly, Stat3 is also important in normal breast development, but this function does not require Src. In addition, β 1-integrin adhesion occurs minimally in differentiated breast epithelium but is upregulated during oncogenic progression and is required for transformation by Src. We therefore hypothesize that β 1-integrin engagement is necessary for Src/Stat3-dependent activation of HGF transcription and breast tumorigenesis. Using 2-D cultures, we showed that Src and Stat3 are strongly activated by adhesion to specific extracellular matrix proteins via β 1-integrins in mammary epithelial cells. We are currently extrapolating these findings to a 3-D culture model in which mouse breast epithelial cells (EPH4) form acini-like spheroids with hollow lumen surrounded by a well-polarized outer layer of cells that is in direct contact with matrix. Under these conditions, Stat3 levels are decreased followed by a reduction in cyclin D1 expression while Src activation remains at a low baseline level over an 8-day culture period. Interestingly, expression of Stat5, which has a reciprocal relationship with Stat3 in breast development and involution, is increased concomitant with elevated β -casein expression. Activation of β 1-integrins by soluble fibronectin alone in this system leads to lumen filling while addition of HGF alone stimulates tubulogenesis without lumen filling. Fibronectin and HGF in combination stimulate tubular outgrowths with lumen filling in epithelial mammospheres, suggesting that integrin adhesion can switch HGF/Met signaling to a transformation phenotype. In a parallel approach, expression of a constitutively active mutant of Stat3 (Stat3C) causes loss of polarity and marked scattering of EPH4 cells, indicative of a transformed phenotype. Future studies using specific inhibitors or shRNA will assess whether integrin adhesion is linked to activation of Src/Stat3 and autocrine HGF expression, and the role of this signaling axis in transformation and metastasis of breast epithelial cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4407; Canadian Institutes of Health Research; and Canadian Breast Cancer Research Alliance.

P69-12: TAMOXIFEN TREATMENT FUNCTIONALLY ALTERS THE RAT MAMMARY STROMA SUGGESTING A ROLE FOR ECM IN TUMOR SUPPRESSION

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Studies show that acquiring genetic mutations required for metastasis is not sufficient to guarantee a successful metastatic event, indicating that the interaction of the tumor cell with its microenvironment is rate-limiting for tumor cell progression. Thus, identifying stromal proteins that determine tumor cell dormancy or progression is critical for the clinical control of breast cancer. Since the functional unit of cancer can be considered the cancer cell plus its microenvironment, we propose that both compartments comprise the response to interventions that reduce risk for cancer progression. To test this hypothesis, we evaluated the effects of tamoxifen, an anti-estrogen targeted to mammary epithelial cells (MEC), on the mammary microenvironment. In this model, mammary extracellular matrix proteins (ECM) elevated by tamoxifen are candidates for tumor suppressors and ECM proteins decreased by tamoxifen are candidates for tumor promoters.

Fifty-four female Sprague-Dawley rats at 70 days of age were randomized into 4 groups and treated daily for 30 days; Gp1: 1.0 mg/kg tamoxifen, Gp2: vehicle control for Gp1, Gp3: 0.5 mg/kg tamoxifen, Gp 4: vehicle control for Gp3. Both low and high dose tamoxifen treatment reduced mammary alveolar development and suppressed MEC proliferation, as measured by morphometrics and BrdU incorporation, respectively.

To determine if tamoxifen affects mammary fibroblasts in addition to the epithelium, mammary fibroblasts were isolated from study rats, expanded in culture to passage 3, and assayed for motility in a transwell filter assay. Fibroblasts from the tamoxifen-treated animals displayed a 3-fold decrease in motility compared to control fibroblasts, indicative of a quiescence. Because fibroblasts are major producers of ECM, we evaluated whether the ECM composition of the gland was altered by tamoxifen. Mammary tissue from tamoxifen-treated rats had decreased levels of fibronectin (FN) by western blot and MMP-2 activity by zymography. Collagen I western blot analysis found elevated levels of fibrillar collagen and a decrease in cleaved collagen in the stroma of tamoxifen-treated rats, suggesting decreased collagen/ECM turnover. IHC analysis of CD68, a macrophage lysosomal marker, demonstrated a decrease in CD68 staining in mammary glands of tamoxifen treated rats. These data demonstrate that tamoxifen treatment results in pleiotrophic stromal changes and indicate that mammary stroma function might also be altered by tamoxifen. Using isolated mammary ECM as substratum, tamoxifen-ECM suppressed transwell filter motility and invasion of MDA-

MB-231 and MCF12A-V12Ras cells, and tumor growth of MDA-MB-231 cells in an orthotopic xenograft model.

To model tamoxifen-induced changes in the mammary ECM using cell culture models, primary mammary fibroblasts from the vehicle and tamoxifen-treated rats were plated for 10 days, fibroblasts removed, and underlying ECM prepared for MALDI-TOF. A loss of FN synthesis by the fibroblasts isolated from tamoxifen-treated rats was observed. Direct treatment of primary mammary fibroblasts with tamoxifen also resulted in reduced fibronectin synthesis. These observations are consistent with our western blot data demonstrating reduced FN in glands of tamoxifen-treated rats compared to vehicle treated. These results will be discussed with respect to known growth-promoting attributes of FN in the mammary gland.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0510; American Cancer Society; and Colorado Cancer Center.

MAMMARY GLAND DEVELOPMENT

Poster Session P70

P70-1: CREATING A BIOREPOSITORY OF “NORMAL” TISSUE AND BIOMOLECULES FOR BREAST CANCER RESEARCH

Connie Rufenberger
Catherine Peachey Fund

Introduction/Objective: In 1998 the National Cancer Institute published the report of the Breast Cancer Progress Review Group (PRG). The Executive Summary of PRG states: “...it is now clear that a more complete understanding of the normal mammary gland at each stage of development—from infancy through adulthood—will be a critical underpinning of continued advances in detecting, preventing, and treating breast cancer.” There are currently few annotated collections of “normal” breast tissue. Research using normal tissue is essential to understanding the causes of cancer, discovering biomarkers, and identifying targets for prevention/treatment. The goal of this project, which was led, designed, implemented, and overseen by the Catherine Peachey Fund, in collaboration with the IU Simon Cancer Center; is to create a large repository of samples of blood, serum, plasma, saliva, and breast tissue from women with no clinical evidence of breast cancer which is collected and stored following the *NCI Best Practices for Biospecimen Resources*.

Description: The Catherine Peachey Fund, Inc., is an Indiana-based, nonprofit, consumer organization that supports breast cancer research. The Amelia Project is the Peachey Fund’s annual scientific meeting, which partners laboratory researchers and clinicians with consumers. The normal tissue bank originated at the Amelia Project. Researchers attending the meeting identified the lack of access to “normal” breast tissue samples as one of the most significant roadblocks to progress in breast cancer research. The shared experience of the group was that “normal” samples were not available in sufficient numbers and that what was available did not meet the required quality standards for reliability. Consumer representatives present realized that this was a need that they could address, and they spearheaded and funded an unprecedented collaboration among consumers, clinicians, basic scientists, volunteers, and donors who took up this challenge. To the best of our knowledge, this is the first biospecimen bank in the world, the purpose of which is to collect specimens from individuals without disease.

It took 3 years to address ethical and IRB issues as well as to develop the necessary infrastructure. Donors fill out a four-page questionnaire of comprehensive medical information. The annotation is linked to the tissue sample through a barcode system. Data is stored in an Oracle database that is HIPAA compliant. The database is available via the World Wide Web to researchers across the world who can query it to determine if there are specimens available that have the potential to facilitate their research.

The Bank is governed by a steering committee made up of a consumer representative, a medical oncologist, and a surgeon. Consumer representatives serve on all standing committees of the Bank.

Summary of Results: The Bank at present contains almost 2,500 specimens including breast core biopsies from 178 women. Outreach to minority populations has been a priority of the Bank, 14% of specimens are from African-American women recruited at Indianapolis Black Expo. Research utilizing the samples is robust and has resulted in poster presentations at the San Antonio Breast Cancer Symposium, publications, and multiple collaborations across the country. The Bank has been recognized by Susan G. Komen for the Cure who has provided a 1-year, one million dollar grant to support the Bank.

This work was supported by The Catherine Peachey Fund, Inc.; Indiana University School of Medicine; and The General Clinical Research Center.

P70-2: MAMMARY GLAND DEVELOPMENT IN IL-10 KNOCKOUT MICE

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Several previous studies suggest that IL-10 may influence mammary carcinogenesis. Mammary epithelial cells and breast cancer cells can both respond to IL-10. IL-10 has been shown to reduce growth and metastases of malignant mammary tumor cells in immunocompetent and immunocompromised mouse hosts. In another mouse model, pathogen exposure increased mammary gland tumor development and an adaptive transfer of IL-10-competent TR cell reduced cancer risk. In the human, an IL-10 gene promoter polymorphism that increases IL-10 production has been associated with decreased breast cancer risk. This polymorphism also increased breast cancer survival after autologous bone marrow transplantation. To test the hypothesis that IL-10 loss will alter the mammary gland in ways that increase breast cancer susceptibility, we are examining the effect of IL-10 gene knockout on mammary gland development and differentiation. Mammary gland comparisons will be made between IL-10 knockout virgin female mice and their wild-type littermates at critical points of mammary gland development, including age d21, d55, d80 and d150 of age as well as 2 days after delivery. We have now compared 5 pairs of mice at d21 (weaning). At this stage, IL-10 knockout did not significantly affect mammary gland structure. In mammary gland wholemount analysis, there were no differences in the number of terminal end buds, the number of ductal side branching, nor the size of lymph node. In the analysis of hematoxylin and eosin-stained paraffin thin sections, there was no difference in leukocyte abundance in the stroma. Initial gross and histologic analysis of the adipose fat pad did

not reveal any significant difference in structure. D21 IL-10 knockout mice and control mice had similar average size of white adipocytes (determined in paraffin sections) and similar area of brown adipose tissue (determined in mammary gland wholemount). To summarize, at day 21 of development, there was no significant difference in gland development between IL-10 knockout and wild-type mice. Ongoing studies are addressing the effects of IL-10 knockout on the development and differentiation of the mammary gland during and post-puberty, time points when leukocytes have been shown to play a role in mammary gland branching morphogenesis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0645.

P70-3: GLOBAL CHANGES IN PATTERNING OF HISTONE MODIFICATIONS MARK MAMMARY GLAND MATURATION AND DIFFERENTIATION

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An early full-term pregnancy has been shown to have a protective effect with regard to the risk of developing breast cancer. It has been suggested that parity-related protection occurs through a change in cell fate, affecting a specific population of mammary epithelial cells. This change in cell fate most likely occurs through an epigenetic mechanism, thus pointing to a direct role for chromatin remodeling in this process. Histone-tail modifications are marks for different chromatin structural states that correlate with development. We hypothesized that there are changes in histone modifications correlating with the protective effect of full-term pregnancy and marking cells with a different fate. To test this hypothesis in an animal model, we (1) determined that unique changes in global histone modification staining patterns and levels in mammary epithelial cells of the mouse mammary gland correlated to full-term pregnancy and (2) tested the significance of global histone modification patterns in samples from hormone pretreated mice and age-matched virgins (AMV) (6 months) that have been treated with/without carcinogen (DMBA). Staining protocols were established for virgin, pregnant, lactating, and involuted mammary gland tissue using antibodies against specific histone modifications. We detected developmental stage-specific nuclear staining patterns for the investigated histone modifications. It showed that the functional differentiation of mammary epithelial cells occurs in concert with a profound reorganization of the chromatin. We did not detect differences in staining patterns or levels between mammary glands after full-term gestation and lactation and age-matched virgins nor did we detect changes correlating with estrogen and progesterone treatment in a tumor prevention model. These findings suggest that the gross changes in histone modification patterning and levels observed during functional differentiation of the mammary gland from virgin to lactation do not persist after lactation has ceased. This illustrates the dynamic nature of the observed changes in chromatin and histone modification patterning and the remarkable plasticity of the mammary epithelial cells. It does not rule out that local chromatin changes, which are not detected by the global analysis employed here, are occurring and important in pregnancy-related protection. Insight in the changes in the compaction of the DNA in breast cells after a full-term gestation can help in understanding the changes that contribute to parity-related protection from cancer. This can lead to new approaches in diagnosis and treatment of this disease that affect millions of women in this country.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0456.

P70-4: MAMMARY INTRADUCTAL FOAM CELLS ARE BONE MARROW DERIVED AND ARE RECRUITED IN RESPONSE TO BOTH PHYSIOLOGICAL AS WELL AS NEOPLASTIC STIMULI

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Ohio State University

Intraductal “foam cells” are the most commonly encountered cells in spontaneous nipple discharge, nipple aspirate fluid, and ductal lavage yet their origin and significance remain a mystery. These cells increase in pregnancy and other conditions of ductal ectasia and obstruction. They frequently surround DCIS and other intraductal proliferations. Our previous immunocytochemical studies with macrophage (CD68, lysozyme), epithelial (cytokeratin, estrogen receptor) and myoepithelial (smooth muscle actin, CALLA, maspin) markers indicated that foam cells are of macrophage lineage and terminally differentiated (negative Ki-67 and PCNA). These foam cells often ingest both endogenous as well as exogenous substances. Because these macrophages are observed only intraductally and because their appearance resembles lactating and vacuolated epithelial cells, their origin had been presumed to be of ductal lining epithelium. However, our previous studies utilizing bone marrow transplantation of donor marrow from female GFP-transgenic C57 black mice into sublethally irradiated female C57 black mice recipients rendered pseudopregnant revealed that the mammary foam cells were of donor bone marrow origin. Mice exhibiting successful bone marrow engraftment of at least 50% donor marrow were identified and made pseudopregnant with a combination of estradiol, progesterone, and estriol (2.5 mg) 21-day release pellets. After this time period and following euthanasia, their mammary fat pads were excised and examined. The presence of GFP-containing intraductal foam cells was found.

As controls for nonspecific phagocytosis of GFP, no free GFP was found within ductal fluid. Furthermore, tail vein injections of GFP-labeled murine lymphocytes and embryonal fibroblasts into female C57 mice made pseudopregnant produced no GFP-containing mammary foam cells. We have extended these initial studies to donor ROSA26 transgenic mice containing the lacZ reporter gene and recipient transgenic mice carrying potent breast cancer oncogenes: (1) the highly penetrant very robust though somewhat unnatural *MMTV-pymT* where polyomavirus middle T is overexpressed and where 100% of mice develop breast cancer with a mean onset of 90 days and (2) the less penetrant but more natural *MMTV-erbB2/neu*. Marrow from each of the ROSA26 donors was transplanted into three transgenic recipients containing either of the aforementioned oncogenes or wild-type controls. Subgroups of recipients having a successful marrow take were rendered pseudopregnant with subcutaneous pellets of estradiol (0.5 mg), progesterone (25 mg), and estril (2.5mg). In both the pseudopregnant as well as nonpregnant oncogene transgenic recipients as well as controls, the mammary foam cells were of donor origin. However, the number of intraductal foam cells was increased in pseudopregnancy 10-fold by intraductal neoplasia 5-fold and by a combination of the two over 25-fold. Ducts with the highest numbers of mammary foam cells exhibited a significantly increased apoptotic index of the adjacent neoplastic cells by TUNEL. These findings suggest a new strategy of delivering therapeutic genes to DCIS, other precancerous lesions, or high-risk ductal epithelium, a strategy that would exploit the omnipresent mammary foam cell, its bone marrow origin, and its chemoattraction to the breast in response to both physiological as well as neoplastic stimuli.

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P70-5: CONTROLLED DIFFERENTIATION OF ADIPOSE CELLS: TOWARD RECONSTRUCTION OF BREAST TISSUE

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A reconstruction method is often sought to restore the aesthetic function of the breast for breast cancer survivors who have undergone surgical treatment. Currently, tissue engineered breast reconstructive options have been limited, due to an inability to produce large volumes of engineered adipose tissue. Adipose cells have been suggested as a cellular basis for these systems; however, the uncertainty behind the mechanisms of adipogenesis in vitro has limited progress. Research has shown that exogenous lipid in culture medium can facilitate both the growth of healthy mammalian cell cultures and the accumulation of cytoplasmic lipid. Thus, the purpose of this study was to investigate the effects of culturing stromal stem cells with lipid-supplemented culture medium. Our long-term objective is to develop cell culturing methods to control the differentiation and proliferation of adipose cells in vitro.

Mouse bone marrow stromal stem cells (D1 cells) of passage 11 were seeded at 20% seeding density in 24-well culture plates. At confluency, cells were supplemented with specific mediums consisting of Dulbecco's Modified Eagle Medium (DMEM) with concentrations of linoleic acid ranging from 0.25% to 12% volume/volume (v/v) (i.e., 1mL linoleic acid/100mL DMEM equates 1%v/v). Control samples were supplemented with either DMEM without additives or DMEM with a normal adipogenic cocktail consisting of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. Cells were analyzed following 7 and 14 days of culture using a PicoGreen assay and a Triglyceride assay to evaluate DNA concentration and triglyceride content, respectively (n=4 per assay). All statistical analyses were performed using SAS 9.1. The least squares mean was used with a significance level of $\alpha = 0.05$.

Studies showed that the addition of linoleic acid to D1 cell culture medium did not negatively affect the DNA concentration (indicative of cell number) for the tested conditions. There was no significant difference ($p < 0.05$) among DNA concentration values for the cells receiving DMEM without additives, DMEM with the normal adipogenic cocktail, or DMEM with any concentration of linoleic acid. Measurements of total triglyceride content indicated that the highest concentration of triglyceride was found in samples supplemented with 12% linoleic acid. The lowest measurements of triglyceride were found in samples supplemented with low concentrations of linoleic acid (0.25-1%). There was no significant difference ($p < 0.05$) found among the samples given DMEM with the normal cocktail and the samples given higher concentrations of linoleic acid (>2%).

Culture medium supplemented with higher concentrations of linoleic acid, i.e., greater than 2%, provided comparable D1 cell total triglyceride content to that obtained when D1 cells were cultured with DMEM supplemented with the normal adipogenic cocktail. These studies are the first step toward our long-term goal of controlling, in vitro, the adipogenic differentiation and proliferation of primary cells. The ability to control adipose cells in vitro will not only provide a means for improved tissue engineering strategies for reconstructing breast defect sites, but will also provide methods that may be employed to further develop 3-D tissue test systems useful for evaluating breast cancer therapeutics and for studying the interactions of breast cancer cells with normal cells.

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P70-6: A CRITICAL ROLE OF ID4 IN MAMMARY DEVELOPMENT

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ID4, a member of the bHLH family, lacks a DNA binding domain and functions primarily to inhibit bHLH proteins. It also suppresses BRCA1 expression. We have found that ID4 plays a critical role in mammary ductal elongation, and this defect is associated with disorganized TEBs and reduced proliferation indices. Furthermore, we have found that ID4 also plays a role in alveolar expansion at pregnancy and in ductal expansion induced by aberrant Wnt signaling. Moreover, we discovered a possible mechanism: ID4 may mediate estrogen and progesterone signaling-induced cell proliferation by suppressing p21, consequently promoting cell cycle progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0457.

P70-7: A HUMAN MAMMARY EPITHELIAL CELL (HMEC) CULTURE SYSTEM FOR THE STUDY OF NORMAL HMEC BIOLOGY AND CARCINOGENESIS

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Our laboratory has had a long-term program to generate human mammary epithelial cells (HMEC) to study normal HMEC processes and how these processes are altered during transformation. Normal HMEC from reduction mammoplasty tissue have been grown in different culture conditions, and then subjected to oncogenic insults to produce in vitro transformed HMEC lines. Widespread use of these HMEC cultures has been facilitated by a website (www.lbl.gov/~mrsgs/mindex.html) and databases for easy storage and retrieval of information on cell inventory and distribution. Our work has led us to propose a molecularly defined model of the senescence barriers encountered by cultured normal HMEC (Garbe et al. *Cell Cycle* 6:1927, 2007) and to hypothesize about how different pathways of molecular alterations may generate the diversity of phenotypes seen in breast cancer cells in vivo. Normal cultured HMEC encounter a first barrier, stasis, which is stress-associated, mediated by the retinoblastoma (Rb) pathway, correlated with increased levels of p16INK4a and telomere length independent. The population doubling (PD) level attained prior to stasis varies greatly depending upon culture conditions. HMEC may overcome stasis by inactivation of the Rb pathway and continue growth until encountering a second extremely stringent barrier due to telomere attrition. Where wild-type p53 is present, this barrier has been termed agonescence and produces a mostly viable growth arrest. If the barrier is approached with non-functional p53, then crisis occurs. HMEC grown in a serum-free medium reach stasis quickly and can show spontaneous silencing of p16, giving rise to the p16(-) post-stasis HMEC (called post-selection) that are commercially available. In serum-containing media, loss of p16 expression has been induced by oncogenic agents but has never been observed to occur spontaneously. We speculate that the stressful conditions induced by the serum-free medium may act like an oncogenic agent. Cultured finite HMEC are also vulnerable to oncogene-induced senescence, which produces a phenotype distinct from stasis, agonescence, or crisis. Overcoming telomere dysfunction to achieve immortality requires reactivation of telomerase activity, which can be present at low levels in pre-stasis HMEC, but is strongly repressed in post-selection HMEC. Combinations of oncogenic events associated with breast cancer can immortalize pre-stasis or post-selection HMEC, and further oncogene overexpression can render the immortalized lines malignant. Our most recent work has delineated simple culture conditions that allow pre-stasis HMEC to grow actively for ~60 PD prior to stasis. Cells with phenotypes indicative of multiple mammary lineages (e.g., basal, luminal, and progenitor) are present. Comparisons of growing and senescent pre-stasis and post-selection HMEC, and isogenic fibroblasts, point out significant differences between normal pre-stasis versus post-selection HMEC and support our model of the distinct senescence barriers. This model is consistent with in vivo carcinogenesis, e.g., overcoming stasis may correlate with early clonal expansion/atypical hyperplasia, whereas cells approaching agonescence show properties similar to DCIS (short telomeres, genomic instability). In total, this HMEC system allows examination of normal and perturbed HMEC biology, the pathways by which normal cells transform, and identification of agents that might promote or inhibit breast cancer progression.

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P70-8: THE ROLE OF HEDGEHOG SIGNALING IN MAMMARY GLAND DEVELOPMENT

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The hedgehog (Hh) signaling pathway is a critical pathway for development and is known to influence proliferation, cell fate, morphogenesis, and stem cell self-renewal.

Mutations activating Hh signaling have been found in a wide variety of cancers, and it is estimated that up to 25% of all tumors display misregulated Hh signaling. Despite the implication of this pathway in breast cancer, little is known about its role in normal mammary gland development. Smoothed (Smo), a seven pass transmembrane protein is the Hh signaling pathway's primary effector and its sole non-redundant member.

To understand the role of Smo in mammary gland development, we performed phenotypic analyses of a conditional mouse model expressing constitutively activated Smo under the control of a mammary gland selective Cre recombinase. We show that activation of the Hh pathway results in increased proliferation starting around 5 weeks of age that leads to a dramatic increase in branching and budding of the mammary gland at around 10 weeks of age. In addition, immunostaining using the ductal markers Na-K-Cl Co-transporter 1 (NKCC1) and aquaporin 5 (AQP5) suggests alterations in cell fate. Finally, these mice exhibit changes in the stroma surrounding the mammary ducts including an increase in collagen deposition.

Gene-profiling studies show upregulation of several genes, including targets of the Notch pathway, in 10-week-old virgin mammary glands from the constitutively active conditional Smo mouse model when compared to control. Surprisingly, canonical downstream Hh target genes like Patched and Gli were not found to be upregulated. These data, in conjunction with recent published studies, suggest that Smo activation in the mammary gland may be acting via a non-canonical mechanism to produce the observed hyper-branched and budded phenotype.

In addition to these gain-of-function studies, experiments are under way utilizing a conditional null Smo mouse model (loss-of-function) to determine if there is a requirement for Hh pathway activation in normal mammary gland development. Preliminary analysis indicates that partial loss of Smo in mammary gland cells leads to impaired ductal elongation and reduced branching.

Future studies will include the generation of mammary gland chimeras to study the possible paracrine interactions between Smo overexpressing and wild-type cells. Additionally, targets of Hh signaling in the mammary gland will be identified by performing cDNA microarray analysis on RNA collected from increasing time-points after Smo activation and following Smo deletion.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0707.

P70-9: MAMMARY EPITHELIAL-SPECIFIC DELETION OF THE FOCAL ADHESION KINASE GENE LEADS TO SEVERE LOBULO-ALVEOLAR HYPOPLASIA AND SECRETORY IMMATURETY OF THE MURINE MAMMARY GLAND

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Integrin-mediated cell adhesion and signaling are required for mammary gland development and functions. As a major mediator of integrin signaling, focal adhesion kinase (FAK) has been implicated to play a role in the survival, proliferation, and differentiation of mammary epithelial cells in previously studies in vitro. To assess the role of FAK in vivo, we created mice in which FAK is selectively deleted in mammary epithelial cells. The mammary gland FAK conditional knockout (MFCKO) mice are viable, fertile, and macroscopically indistinguishable from the control littermates. In virgin MFCKO mice, mammary ductal elongation is retarded at 5 weeks of age but reaches the full extent by 8 weeks of age compared to the control mice. However, the MFCKO females are unable to nurse their pups due to severe lobulo-alveolar hypoplasia and secretory immaturity during pregnancy and lactation. Analysis of the mammary epithelial cells in MFCKO mice showed reduced Erk phosphorylation, expression of cyclin D1, and a corresponding decrease in proliferative capability, compared to the littermate controls. In addition, phosphorylation of STAT5 and expression of WAP are significantly reduced in the mammary glands of MFCKO mice, suggesting defective secretory maturation in these mice. Therefore, the combination of the severe lobulo-alveolar hypoplasia and defective secretory differentiation is responsible for the inability of the MFCKO females to nurse their pups. Together, these results provide strong support for a role of FAK in the mammary gland development and function in vivo.

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P70-10: Fog2 FUNCTION IN THE MAMMARY GLAND DEVELOPMENT

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During embryogenesis GATA proteins are essential regulators of the development and differentiation in many tissues. In the adult the pivotal role for GATA family proteins in maintaining the normal (non-transformed) state of mammalian cells is corroborated by the recent findings of mutations or methylation of GATA genes both in primary cancers and tumor lines. Mutations of GATA1 in Down syndrome-associated myeloid leukemia provide support for the hypothesis that mutations of GATA genes occur early

in cancer. In humans GATA proteins are already implicated as potential tumor suppressors in various tissues, including breast. Microarray profiling studies determined that the highest expression of both GATA3 and ESR1 (estrogen receptor alpha) is seen in tumors associated with the most favorable survival outcomes while the lowest expression of GATA3 is detected in tumor subtypes showing the worst outcomes. At this time, genes and pathways that are regulated by GATA3 in the mammary gland are not well defined. We have previously established a requirement for FOG (Friend of GATA)-2 co-factor during mouse development. Here we used a reporter line of mice to characterize Fog2 expression in the mammary gland. We report that in the murine mammary gland Fog2 gene expression is up-regulated upon pregnancy and lactation with prominent expression in the epithelial cells of the gland during post-lactational regression (involution). We have generated animals with a mammary-specific loss of the Fog2 gene and report here that WapCre-mediated excision of the Fog2 gene leads to the accelerated involution of the gland despite diminished levels of the remodeling enzymes. Importantly, the levels of several genes linked to the control of cancerous transformation in the breast (Esr1, Prg, Foxa1) are significantly reduced upon Fog2 excision. Our data implicate GATA3/FOG2 complex in the transcriptional control of gene expression in mammary gland and the maintenance of epithelial cell differentiation and survival.

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P70-11: USE OF A UNIQUE HUMAN BREAST TISSUE ENGINEERING MODEL TO DETERMINE THE ROLE OF ENVIRONMENTAL XENOBIOTICS IN AFRICAN AMERICAN BREAST CANCER ETIOLOGY

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African American (AA) women have a lower incidence rate of breast cancer (BC) than white women. Despite this, AA women are more likely than white women to die from the disease. BC survival 5 years after initial diagnosis is only 74% among AA women compared to 88% among white women. This difference has generally been attributed to socioeconomic factors such as the lack of health insurance, lower incomes, and unequal access to medical care. In addition, cultural differences exist, such as attitudes toward breast feeding (a factor that decreases BC risk). However, increasing evidence exists to support the hypothesis that there may be intrinsic biological differences between the breast tissues of white and AA women. One of the hormonal issues under study now is precocious puberty, which is increasing in the United States and is most dramatic in AA girls. Since breast development may be related to BC etiology, we have begun a study of environmental xenobiotics on a unique collection of cell lines generated using a tissue engineering system. We have established primary HMEC cultures and cell lines from 36 breast reduction mastoplasty tissues, and 9 of the tissue donors were AA and matched in SES with the white women. These cell lines maintain a normal karyotype after 20–30 passages, presumably due to the maintenance of a stem cell compartment in vitro. In addition, the original explants uniquely form functional, organotypic ductal structures in culture.

We have found that the more children a woman had, the less likely her breast tissue was to form ductal structures in vitro, and that premenopausal breast tissue was more likely to form ductal structures than postmenopausal tissue. Race contributed as a modifying factor in the ability to form ductal architecture in vitro with SES matched AA tissue demonstrating more robust and earlier spontaneous differentiation in vitro than samples from white women.

Several common environmental polycyclic aromatic hydrocarbons (PAHs) have recently been purported to be associated with BC incidence. One ubiquitous chemical in this category is benzo[a]pyrene, which is a product of smoking and the burning of fossil fuels. A second agent, bisphenol A, is associated with polycarbonate plastics. Bisphenol A, originally developed for use as a synthetic estrogen, is now an extremely high-volume chemical with over 6 billion pounds manufactured annually. Ninety-five percent of U.S. girls show bisphenol A in their urine.

We have used one nondiseased cell line and one unprecedented ductal carcinoma in situ (DCIS)-derived cell line to establish cytotoxic dosimetry for an activated metabolite of benzo[a]pyrene, benzo[a]pyrene diol-epoxide (BPDE), and for bisphenol A. Multiple exposures to BPDE caused a change in the karyotype of our DCIS cell line from pseudo-tetraploid to hexaploid within 3 months. Several nondiseased breast cell lines (from both AA and white patients) are currently being treated with BPDE and bisphenol A to determine whether nondiseased cell lines can be altered karyotypically in a relatively short length of time by environmental doses of these chemicals. The interaction of these chemicals with metabolically distinct populations of different ancestry may begin to explain the prevalence of earlier onset, poorly prognostic triple-negative BC in AA versus white women.

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P70-12: CELL FUSION IN NORMAL DEVELOPMENT AND ABNORMAL GROWTH

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Background and Objectives: Cancer mortality is due mainly to the ability of tumor cells to disseminate and grow throughout the organism. It has been speculated that metastatic ability could be acquired through fusion of cancer cells with normal cells that have the ability to migrate and settle in different organs. Evidence for the increased propensity of cancer cells to form cell hybrids and for the increased malignancy of the hybrids has also been obtained. Despite these findings, the role of cell fusion in the evolution of tumors is generally unknown. This project examines the hypothesis that cell fusion between normal and tumor cells occurs with a detectable frequency in an insulin-like growth factor 2 (Igf2) transgenic mouse model of metastasizing mammary tumors, and that metastatic variants may be created through such fusion.

Brief Description of Methodologies: We have set out to detect cell fusion between normal and tumor cells by activating a color marker in fused cells of aggregation chimeras using the Cre-lox system. To achieve this goal it is necessary to: (1) Introduce the Igf2 transgene into a mouse line that is homozygous for a ubiquitously expressed Cre transgene (ACTB-cre). (2) Introduce an X-linked green fluorescent protein (GFP) (which allows sexing of early embryos) into both the mouse line containing Igf2/ACTB-cre and a line that contains a lacZ gene kept inactive by a loxP-flanked DNA fragment (3) Produce aggregation chimeras between Igf2 transgene positive (Tr+) or transgene negative (Tr-) female embryos homozygous for ACTB-cre and female embryos homozygous for the inactive lacZ gene (4) Detect cell fusion through the Cre-

mediated activation of lacZ in chimeras that develop metastasizing mammary tumors. Progeny is genotyped by PCR and Southern blotting. Aggregation chimera production and lacZ staining are carried out by standard techniques.

Results to Date: Igf2 transgenic mice that express the transgene under the control of the H19 enhancers and develop metastasizing mammary tumors have been produced in our laboratory. These mice have been crossed with the FVB/N-Tg(ACTB-cre) 2MrJ mice (ACTB-cre) to generate a line that contains the Igf2 transgene and is homozygous for the ACTB-cre transgene. Mice that carry an X-linked GFP transgene [strain TgN(GFPX)4Nagy] or the inactive lacZ gene [B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J] were obtained from the Jackson Laboratory and congenic lines carrying these transgenes on an FVB/N background have been produced. GFPX mice have been crossed with Igf2/ACTB-cre and lacZ mice to produce males with a GFP- marked X chromosome. Aggregation chimeras between Igf2/ACTB-cre and lacZ embryos are being produced, bred, and monitored for tumor development and cell fusion. The results of this analysis will be presented.

Conclusions: The conclusions from this study await the completion of the chimera analysis. The proposal examines a novel mechanism for the generation of metastatic variants in an animal model of breast cancer. Knowledge of the frequency of cell fusion in the primary tumor and its metastasis has important implications for understanding the mechanisms of tumor progression. If cell fusion is demonstrated to play a role in the emergence of metastatic variants, new approaches for the prevention or treatment of metastatic disease may become possible.

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TUMOR PROGRESSION II

Poster Session P71

P71-1: CAPTURE OF CIRCULATING CANCER CELLS USING MICROFLUIDIC SYSTEMS

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The use of a microfluidic system to capture metastatic cancer cells from suspension has been studied. Here capture occurs on microchannel surfaces that contain an immobilized cadherin ligand, which specifically binds the target cells, and capture has been studied under both no-flow and flow conditions. Breast carcinoma cells that metastasize to the circulation, in contrast with normal epithelial cells, typically downregulate E-cadherin and can upregulate N-cadherin. Hence, the cadherin system provides a natural tool to select metastatic tumor cells circulating in the blood.

The microchannel fabrication process starts with patterning a 3000Å-thick thermal oxide layer on a 2" silicon wafer. A TMAH solution is used to etch channels about 100 µm deep, and the oxide mask is stripped away. The substrate is then re-oxidized forming a 1 µm-thick silicon dioxide layer. Separately, a glass cover slip, with inlet/outlet holes, is made to place over the etched silicon base and forms the upper surfaces of the channels. The cover slip is incubated with PEG solution to prevent nonspecific binding on the upper channel surface. UV glue, applied with a fabric brush, is used to bond the cover slip and the etched-silicon base in a contact mask aligner. Bottom surfaces within the channels are then derivatized as follows. The hydroxyl groups on the oxide surface are silanated in 1% (vol./vol.) 3-aminopropyltriethoxysilane (APTES)-acetone solution followed by activation in 2% (vol./vol.) glutaraldehyde in phosphate buffer saline. Protein G is next incubated on the activated surface. Finally, anti-N-cadherin IgG or E-cadherin IgG is immobilized on the protein G layer through binding of the Fc region.

To demonstrate cell capture, N-cadherin-expressing test cells have been incubated in microchannels coated with either anti N-cadherin or normal IgG antibodies at room temperature for 15 min in calcium-free media with 0.1% BSA. After standard washing, 80% of the cells initially present in the channel are captured on the anti-N-cadherin surface under no-flow conditions (Figure 1). Only 10% are captured on the mscIgG surface. Increasing the incubation time from 5 to 15 min increased the percentage of captured cells from 10% to 80%. Once strong binding is established, flow induced shear stress with average velocity of up to 3 mm/s failed to dislodge the captured cells. In dynamic conditions, that is cell capture from flowing suspensions, cells with a translation velocity as low as 0.1 mm/s have been observed to roll on the derivatized surface though no cells have been captured at even these modest translation rates.

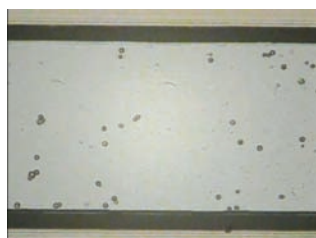


Figure 1. Cancer cells captured in a microchannel, 100 µm deep and 1 mm wide.

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P71-2: GENE EXPRESSION PATTERNS WHICH DISTINGUISH BASAL B (MESENCHYMAL) HUMAN BREAST CANCER CELL LINES FROM BASAL A OR LUMINAL ARE EXHIBITED BY PURIFIED HUMAN BREAST CANCER STEM CELLS. EVIDENCE OF EPITHELIAL MESENCHYMAL TRANSITION IN CLINICAL SPECIMENS

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Epithelial mesenchymal transition (EMT) has long been associated with breast cancer cell invasiveness and evidence of EMT processes in clinical samples is growing. Genome-wide transcriptional profiling of increasingly larger numbers of human breast cancer (HBC) cell lines have confirmed the existence of a subgroup of cell lines (termed "Basal B"/Mesenchymal) with enhanced invasive properties and a predominantly mesenchymal gene expression signature, distinct from subgroups with predominantly luminal (termed "Luminal") or mixed basal/luminal (termed "Basal A") features (Neve et al., *Cancer Cell*, 2006). Expression of genes in a literature-derived EMT signature (EMT SIG) showed an EMT (positive) signature in the Basal B lines, and an EMT (negative) signature in the Luminal lines. The coordinate expression of EMT SIG genes in these cell lines supports a role for EMT in the differences between these HBC subtypes and an important biological role for EMT in different breast cancer subtypes. Further analysis of these data has implicated specific transcriptional programs in the HBC EMT, with Zeb1 and TCF-4 showing highest selective expression in the Basal B group. Snail 2 and TWIST were overexpressed in Basal B lines, but also in some Basal A and Luminal lines, respectively. We also identified potential new markers/regulators

of EMT in the Neve dataset. We have examined these associations further in the HBC cell line PMC42ET and its epithelial counterpart (PMC42LA), which undergo EGF-induced EMT (Ackland et al., *Lab Invest*, 2003). Each line displays typical morphology, gene expression and immunohistochemistry with regard to epithelial (CDH1, occludin, claudin-1, mucin-1, with membranous beta catenin) and mesenchymal genes (vimentin, fibronectin, MMP-2, Snail1, Snail2, Zeb1/delta EF1). Transfection and siRNA analysis of several EMT regulators confirmed a pre-eminent role for Zeb1 in the PMC42 HBC system. Other relationships have also been examined further in the context of the PMC42 cell system and complement our recent work demonstrating that breast cancer-associated fibroblasts selectively stimulate EMT-related changes in the PMC42 cell line in 3-dimensional culture (Lebret et al., *In Vitro Cellular & Developmental Biology*, 2007 and *Breast Cancer Research*, 2007). Strong concordance was also found between gene products associated with the EMT phenotype in HBC cell lines and cells sorted from clinical specimens (Shipitsin et al., *Cancer Cell*, 2006), consistent with EMT attributes being advantageous for stem cells. Loss of CD24 is a defining attribute of the Basal B subgroup, where the level of CD24 correlates tightly with degree of mesenchymal gene expression. Of 60 gene products correlating tightly with CD24 status in Basal B cell lines, 27 were identified as differentially expressed in the Shipitsin data set ($P < 0.0001$). These new data confirm and extend the importance of EMT and the value of analyzing established HBC cell lines for new leads in this area. At a time when EMT remains somewhat controversial as a clinical entity, evidence of EMT-like gene expression in cells purified from breast cancers adds valuable support to a growing number of reports associating EMT parameters with poor clinical outcome in breast cancer.

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P71-3: CHROMOSOME ENGINEERING AND SLEEPING BEAUTY INSERTIONAL MUTAGENESIS TO IDENTIFY 1p TUMOR SUPPRESSORS AND OTHER GENETIC EVENTS IN BREAST CANCER PROGRESSION

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Background and Objectives: Breast cancers driven by the oncogenes HER2 and c-Myc display a range of biology and response to therapy, presumably due to genetic heterogeneity. We have initiated forward mutagenesis screens in mice to identify genes involved in tumor progression and to present novel drug targets. Since many poor-prognosis breast tumors display genomic loss of the chromosome 1p arm, a long-term objective is to identify genes mapping to 1p that contribute to malignant progression in breast cancer.

Methods: We have combined the Sleeping Beauty (SB) insertional mutagenesis system with transgenic mouse models of breast cancer. In this system, T2/Onc mutagenic transposons are mobilized by a trans-acting SB transposase (SB10 or SB11), inserting transposons randomly throughout the mouse genome and activating or inactivating host genes. Cells harboring insertions in genes that contribute to tumor development are clonally expanded during tumor growth. Genes harboring insertions in multiple tumors are considered common insertion sites (CIS) and represent possible oncogenes or tumor suppressors. We combined T2/Onc with mouse models for breast cancer driven by the MMTV-HER2/*neu* and MMTV-*c-myc* transgenes, which express these oncogenes in mammary tissue and develop focal mammary tumors. Simultaneously, we are generating mice in which a 60Mb syntenic region on mouse chromosome 4, corresponding to human 1p, is flanked by loxP sites and can be deleted in mouse mammary tissue using the Cre recombinase. Our long-term goal is to combine this system with SB by generating a large region of hemizygosity and using SB to provide a readily identifiable deactivation of the second allele of any tumor suppressors.

Results to Date: We have generated 27 tumors in an MMTV-HER2/*neu* background carrying SB10 and T2/Onc. We have utilized 454 pyrophosphate sequencing to recover 1,891 sequence reads from 11 of these tumors (average 171 per tumor) and are analyzing these sequences for genomic location and CIS among multiple tumors. Our expanded cohort of mice utilizes a more-active transposase construct (RosaSB11) and alternative transposons (T2/Onc2 and T2/Onc3) to identify a broader range of genes and includes mice carrying the MMTV-*c-myc* transgene. Mice carrying one line of the T2/Onc2 transposon (on chromosome 4) show a preference for integration into the human 1p syntenic region, as T2/Onc transposons have a bias to re-integrate into the same chromosome as the transgene concatemer. Mice from these crosses are being monitored for tumor onset. Additionally, we isolated and cultured tumor cells carrying T2/Onc and introduced SB11 transposase via viral transduction. These cells can be transplanted into mammary fat pads of recipient mice to identify mutations involved in late stages of tumorigenesis.

In parallel, we have modified a loxP targeting vector to include an epitope tag to assess in vivo recombination efficiency and have removed or re-oriented elements that may interfere with neighboring genes. This construct will allow us to target a loxP site to the mouse chromosome 4 telomere without disrupting any host genes prior to somatic recombination.

Conclusions: Insertional mutagenesis and chromosome engineering should identify novel drug targets and illuminate mechanisms of tumor development.

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P71-4: EPITHELIAL TO MESENCHYMAL TRANSITION IN BREAST CANCER IS ASSOCIATED WITH RESISTANCE TO THERAPY AND A CANCER STEM CELL-LIKE PHENOTYPE

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Epithelial to mesenchymal transition (EMT) and acquisition of cancer stemness are the two key steps for tumor progression, metastases development, and relapse of the disease. While self-renewal of cancer stem cells (CSCs) is responsible for tumor maintenance, EMT confers an invasive and migratory phenotype. Both EMT and the CSCs phenotype have been linked to the development of chemoresistance (Shah et al, *Anti-Cancer Drugs* 2007, 18:371-375; Dean et al, *Nature Reviews* 2005, 5:275-284). Since relapse of breast cancer is explained by resistance to conventional therapies, the goal of this study was to determine if mesenchymal breast cancer cell lines shared a CSC phenotype and the ability to resist chemo- and radiotherapy. The established mesenchymal cell lines (M1, M2, M3, and M4) were from neu-antigen-negative variant relapse breast tumors that had developed following immune-mediated rejection of neu-positive epithelial tumors within parental nontransgenic FVB/N mice through EMT (Knutson et al, *J. Immunol.* 2006,177:1526-1533). Quantitative flow cytometric analysis with MESF (molecules of equivalent soluble fluorochrome) beads showed that mesenchymal cell lines were CD24^{low}CD44^{high}ESA+, as observed in human breast CSCs (Al-Hajj et al, 2003; *PNAS*, 100:3983-3988). Furthermore, all of the mesenchymal cell lines were highly tumorigenic as compared to the originating epithelial cell lines. Western blot analysis showed a higher expression of breast cancer resistance protein BCRP (ABCG2) in mesenchymal cell lines. BCRP has been associated with efflux of mitoxantrone, topoisomerase-I-inhibitors, methotrexate, and some ErbB1 inhibitors. Chemoresistance assays with mitoxantrone and lapatinib (1 μ M) and clonogenic radiation assays after short courses of fractionated irradiation (0, 4, 8, 16, and 32 Gy) showed that mesenchymal cell lines were highly resistant to treatment compared to the parental epithelial cell line. Therefore, these results suggest that EMT may lead to breast tumors with a CSC-like phenotype and ability to resist conventional breast cancer therapies. Understanding the cellular and molecular characteristics of tumorigenicity and resistance to therapy could potentially lead to identification of new therapeutic targets.

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P71-5: MicroRNA AND BREAST CANCER PROGRESSION

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We hypothesized that certain miRNA species are differentially expressed in normal breast epithelium and breast cancer cells. Accordingly, we isolated RNAs from human breast cell lines MCF7, BTK20, T47D, HBL100, MDA-MB-231, MDA-MB-468, and MCF10A. Total RNA isolation was done with Trizol (Invitrogen) as described by the manufacturer. RNA labeling and hybridization were done as described previously using commercial kits (Starfire, IDTDNA Inc. or Ambion's MirVANA).

We used miRNA microarrays (mirVANA v2, Ambion) to evaluate miRNA expression in these breast cancer cell lines. As a control, we used RNA from three different batches of normal mammary epithelial cells (HMEC). To identify miRNA with difference in expression between cancer and normal cells, we used ANOVA (at $p < 0.05$). To confirm results obtained by microarray analysis, we performed Northern blots on some of the differentially expressed miRNAs. In particular, we analyzed mir-145, mir-21, mir-125b, and mir-155 expression. Among those four miRNAs, mir-145 and mir-125b showed decrease of expression and mir-21 and mir-155 showed increase of expression in breast cancer cells in comparison with normal breast epithelium cells (HMEC). When RNAs from ten primary breast cancer samples were analyzed, the conclusions on mir-145, mir-21, mir-125b, and mir-155 expression remained the same as with breast cancer cell lines, suggesting that observed changes occur in primary tumors and not during breast cancer cells adaptation to growth in tissue culture.

We analyzed the predicted targets of the most significantly down- (mir-145 and mir-125b) and up-regulated miRNA (mir-21 and mir-155) to ascertain the potential effects of these miRNAs alterations in expression using three commonly used algorithms: TargetsScan, miRanda, and PicTar (Lewis et al., 2003; Enright et al., 2004; Krek et al., 2005). We proceeded to identify targets that were identified by at least two out of three programs. We expected that targets of down-regulated miRNAs might include potential oncogenes and/or other positive growth regulators. Indeed, among putative targets of mir-125b, we found oncogenes (Ets1, Akt3, and Yes), growth factor receptor FGFR2 and several members of the mitogen-activated signal transduction pathway (MAPK10,

MAPK11, MAPK14). Among predicted targets of mir-145, we found oncogenes FOS, MYCN, YES, and FLI1. Additional targets include cell-cycle proteins, such as cyclin D2. For the upregulated in breast cancer miRNAs (mir-21 and mir-155) we expected that some negative growth regulators (such as tumor suppressors and negative growth regulators) might be affected. Indeed, we found that known negative growth regulator, TGF-beta, was a potential target of mir-21. Tumor suppressors APC and SOCS1 and negative cell cycle regulator Wee1 were predicted to be the targets of mir-155. Hypoxia-inducible transcription factor HIF1A was also among mir-155 targets. Of particular note was the observation that mir-145 was progressively reduced from normal breast to cancers with higher tumor stage. Reciprocally, mir-21 was progressively upregulated in the same samples, suggesting that, perhaps, changes in these two miRNAs regulation contribute to the onset or severity of breast cancer phenotypes.

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P71-6: GENETIC DISSECTION OF THE ROLE OF HEPARAN SULFATE IN MAMMARY TUMOR PROGRESSION

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Background and Objectives: Heparan sulfate (HS) exists mainly as cell surface and extracellular matrix molecules and are functionally involved in various biological processes. Considering its interactions with a number of growth factors/cytokines, it is likely that HS plays an important role in cancer development and progression. Two signaling molecules that have strong implications in human breast cancer, namely Wnt1 and neuregulin (the Ig-domain containing isoforms), bind and functionally modulated by HS. Factors known to affect invasion, metastasis, and tumor angiogenesis, such as matrix metalloproteinases, VEGF, and endostatin, also interact with HS. Despite this wealth of data, our understanding of the mechanisms by which HS influences tumor cell behavior in vivo is still fragmentary. One of the important unknowns—what is the overall physiological effect of HS on tumor development and progression. Further compounding the issue is that HS is produced not only by tumor cells themselves but also by stromal cells within tumors. The field needs advanced animal models that not only closely mimic clinical cancers but also allow precise dissection of HS function in different cell types.

Methodologies: Our experimental tool is the conditional Ext1 allele. The key glycosyltransferase for the HS biosynthetic process is the GlcNAc/GlcA copolymerase encoded by the Ext1 gene. Genetic and biochemical studies have established that EXT1 is absolutely essential for HS biosynthesis. We have created loxP-modified Ext1 allele, from which conditional Ext1 knockout mice can be generated by crossing with Cre transgenic mice targeted to various tissue and cell types. In this project, conditional ablation of the Ext1 gene will be combined with polyoma middle T antigen (PyMT)-dependent de novo mammary tumorigenesis models. We will produce MMTV-Cre;KFS2MT6;Ext1^{lox/lox} animals (KFS2MT6 is a PyMT transgene that is activated by Cre-mediated excision of the STOP cassette). MMTV-Cre will activate PyMT expression and disrupt Ext1 concurrently in the mammary epithelium. Therefore, this system will allow us to examine specifically the role of tumor cell autonomous HS. We will also examine PyMT-induced mammary tumorigenesis in the HS-deficient stromal environment. For this, Ext1 will be disrupted specifically in stromal fibroblasts, without interfering HS synthesis in tumor cells, using the FSP1-Cre transgene. This experiment will allow us to determine the role of stromal cell-derived HS in tumor progression. In both experiments, mammary tumor progression will be analyzed in terms of growth, angiogenesis, invasion, and metastasis. Microarray analysis will be performed on tumor RNAs to gain insight into the difference in intracellular signaling between Ext1 null and control backgrounds.

Results to Date: We have completed backcrossing all necessary lines to C57BL/6 and are currently breeding experimental and control groups for the tumor analyses.

Conclusions: Considering its clear implication in tumor growth and progression, HS is an emerging therapeutic target in breast cancer. Yet our current concept on the role of HS in tumor development is derived mostly from the observations obtained from non-physiological experimental models. This project would have a direct impact on this issue by defining the physiological role of HS in mammary tumor development and progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0461.

P71-7: MATRIX METALLOPROTEINASES IN BREAST CANCER PROGRESSION

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Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes capable of degrading all components of the extracellular matrix. In addition, they have potent "shedase" activity, solubilizing biologically active molecules and permitting communication between tumor cells and the surrounding microenvironment. Small

molecule inhibitors of MMPs (MMPIs) were tested as cancer therapy in various tumor types but were ineffective. Many issues surround the MMPI clinical trials, but include a lack of information on the specific profile of MMPs that contribute pro-tumorigenic effects and those that have protective effects. To address this issue in breast cancer progression, the role of specific MMP family members was tested using MMP-null mice and a mouse model of breast cancer, the MMTV-Polyoma virus middle T-antigen (PyVT) model. The lack of MMP-3, 7, and 9 had no effect on the onset, multiplicity, or size of mammary tumors that developed in PyVT mice. Lack of MMP7 also had no impact on lung metastasis. MMP3 deficiency led to a 75% reduction in lung metastasis while lack of MMP9 resulted in an 81% reduction in the number of metastatic lesions on the lung surfaces (Figure 1). The major contribution of host-derived MMP9 to lung metastasis was confirmed using a PyVT-derived cell line in an experimental metastasis assay with wild-type and MMP9-null mice. The experimental metastasis model also allows exploration of tumor cell interactions with the microenvironment using *in vivo* imaging. We have recently begun using two-photon microscopy to examine the kinetics of tumor cell extravasation and growth within the liver (Figure 2). Further analysis of the roles for different MMP family members in the process of metastasis will clarify whether MMP inhibition remains a viable therapeutic strategy.

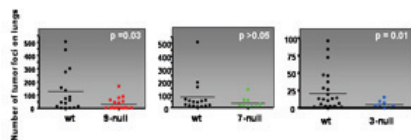


Figure 1. Effects of MMP ablation on lung metastasis in the PyVT model

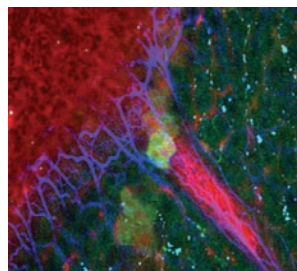


Figure 2. Metastasizing tumor cell within vasculature of liver

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4448 and National Cancer Institute.

P71-8: SYSTEMATIC SCREENING OF THE REGULATORS FOR EPITHELIAL-TO-MESENCHYMAL TRANSITION IN BREAST CANCER

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One essential step for breast cancer metastasis is epithelial-to-mesenchymal transition (EMT), which allows the primary tumor cells to acquire the ability to migrate and invade tissue. The main players promoting the EMT process; however, remain largely unknown. In this study, we will systematically identify the EMT regulators by high-throughput screenings using a GFP reporter system. To establish the reporter cells, we constructed an expression vector that expresses GFP under the control of E-cad promoter. Our data showed that Twist was able to inhibit the GFP expression driven by E-cad promoter. We established two stable cell lines by transfection of E-cad-GFP plasmids into MCF10A and MDA-MB453 cells, respectively. These two cells will be used to systematically screen the regulators for EMT by transfection of genome-wide siRNA oligo library. Currently, we are determining which liposome is suitable for reverse siRNA transfection by testing 10 liposome reagents from different companies. We will then adapt the robot system to transfect the individual siRNA oligos (covering 2,200 genes) into the reporter cells and GFP intensity from immunofluorescent microscope will be used as the readout for our screening. The identified EMT regulators via this screening will be presented in the meeting in June. Since EMT is the initial and one of the most critical steps during the metastatic process, we believe that our studies will provide the first step toward the development of novel therapeutic strategies for the specific treatment on metastatic breast cancer, and as a result, lead to the significant reduction of breast cancer death.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0652.

P71-9: THE ROLE OF ERBP IN BREAST CANCER PROGRESSION

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Metastasis, a process during which primary tumor disseminates into distal sites, likely occurs when primary tumor cells obtain additional genetic or epigenetic alteration. ERBP (estrogen receptor binding protein) is an estrogen receptor binding protein that potentiates the transcriptional activity of estrogen receptor. Unlike most coactivators that interact with AF2 domain of estrogen receptor, ERBP interacts with the DNA binding domain of estrogen receptor. The altered expression of ERBP could promote the metastasis through enhancing the expression of genes that are regulated by estrogen and are involved in the breast cancer metastasis. By overexpressing ERBP in breast

cancer cells, we found ERBP overexpression enhanced the migration and invasion capability of tumor cells. ERBP overexpression also promoted the tumor formation in nude mice. We identified 8 estrogen inducible genes that were upregulated by ERBP overexpression. Finally, we found that expression of ERBP is increased in about 30% of breast cancers.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0583.

P71-10: CD151 AFFECTS ErbB2-STIMULATED MAMMARY TUMOR PROGRESSION

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Background and Objectives: Our research addressed the importance of the CD151 protein, which is associated with malignancy in breast cancer and other types of cancer. Others had found that CD151 knockout mice show normal development, but deficiencies in wound healing. This supported our prediction that CD151 targeting will affect pathological, but not normal physiology. Furthermore, tetraspanin-type proteins (e.g., CD151) had never before been targeted in the context of breast cancer. We hypothesized that the CD151 protein plays a critical role during mammary tumor progression and also carries out vital functions on mammary stem cells. Our proposed experiments were aimed at providing a definitive test of this hypothesis. Also we wanted to determine the feasibility of targeting CD151 to disrupt mammary tumor progression.

Brief Description of Methodologies: Using a mouse model of mammary carcinogenesis, we first aimed to confirm that CD151 is indeed critical for breast tumor progression *in vivo*. To achieve this we generated CD151-null mice, and then crossed them with FVB-Tg(MMTV-ErbB2) mice (Jackson Laboratories), a transgenic strain expressing activated an *ErbB2* (*c-neu*) oncogene that induces mammary hyperplasia and tumorigenesis. Our next goal was to isolate mammary tumor cells, from wild-type and CD151-null mice, for the purpose of understanding the role of CD151 in these mammary cells.

Results to Date: We have successfully generated CD151-null mice and crossed these (as well as CD151-positive mice) with FVB-Tg(MMTV-ErbB2) mice that express amplified *ErbB2* (*c-neu*) oncogene. Initially, we monitored tumor progression in 11 MMTV-ErbB2 mice that express CD151. These began to show mammary tumors by 200 days after birth. By contrast, 6 MMTV-ErbB2 mice that lack CD151 did not begin to show tumors until 300 days after birth. Hence, tumor appearance was substantially delayed when CD151 was absent. Due to the small numbers of mice involved, these results are not statistically significant. A larger experiment is under way, which involves 30 mice expressing CD151 and 30 mice not expressing CD151. Results from this experiment should be available in early 2008. In addition, multiple mouse mammary tumor cell lines have been established from CD151-null and CD151-wild-type mice. Furthermore, ablation of CD151 impaired migration, invasion, adhesion, signaling, and EGFR collaboration in human mammary epithelial cells.

Conclusions and Potential Impact on Breast Cancer: We have obtained multiple lines of evidence supporting our main conclusion that CD151 plays a key positive role during breast cancer progression *in vivo*. Not only did absence of CD151 delay tumor progression in a mouse model *in vivo*, but also the absence of CD151 also affected several mammary tumor cell functions *in vitro* (migration, invasion, etc.). Together our results point to CD151 as being a novel target, which may possibly be used for treating breast cancer, with possible advantages over inhibition of laminin-binding integrins.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0580.

P71-11: AN IN VITRO SYSTEM FOR SIMULATING THE TUMOR MICROENVIRONMENT

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The spread, or metastasis, of breast cancer to vital organs and tissues is responsible for the mortality of the disease. The progression of a tumor to an aggressive metastatic form is the result of a cascade of genetic, proteomic, and metabolic changes. We propose that a key trigger in the development of metastasis is the fluctuation in oxygen content of the tumor microenvironment. These fluctuations may be relatively rapid, as observed in untreated tumors, or slower such as those observed as a result of therapy. We hypothesize that tumor hypoxia, and most importantly, subsequent periods of reoxygenation condition cells to survive the stresses inherent in its ability to metastasize. Hypoxia/reoxygenation is difficult to study since current *in vivo* and *in vitro* models of this process do not have the flexibility to systematically control critical experimental variables nor the ability to dynamically monitor the resulting metabolic changes. This 1-year Concept Award funded work to develop a novel perfusion system that supports the growth of human breast cancer cells and allows rapid changes in oxygen content, pH, and/or metabolic substrates. The resulting multichannel system incorporates membrane oxygenators and voltage-gated switches to precisely control the environment of cells in monolayer or three-dimensional culture. Some aspects of this

system are still undergoing design changes to optimize flow characteristic through the cell growth chamber. In all designs, oxygen content can be controlled to closely mimic the rapid, periodic fluctuations observed in human tumors. We have begun to analyze the effects of chronic and intermittent hypoxia on cell growth, glucose and glutathione metabolism, and tumor invasiveness. Two breast cancer cell lines, the estrogen-receptor (ER)-positive MCF-7 line and ER-negative MDA-MB-231 cell lines were used as models of a relatively noninvasive, and highly invasive cell line, respectively. Under chronic hypoxia (1% O₂), the MCF7 cell line shows slower growth and reduced contents of the primary cellular antioxidant glutathione. The MDA-MB-231 line also grows slower under hypoxia but may take longer to alter its glutathione content. Our initial studies also show that peroxide induced oxidative stress leads to increased metastatic potential as measured by an in vitro invasion assay. Our studies suggest that hypoxia can affect cellular antioxidant levels likely through an increase in oxidative stress. Studies are continuing in our laboratory to compare the levels of stress induced by chronic and acute hypoxia and how this may influence tumor cell invasiveness.

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P71-12: ANNEXIN II INTERACTION WITH tPA ON BREAST CANCER CELL SURFACE FACILITATES PLASMIN GENERATION WHICH MAY BE INVOLVED IN NEOANGIOGENESIS AND AGGRESSIVE PHENOTYPE OF CANCER

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Many advanced human tumors including breast cancer overproduce plasmin that is known to promote angiogenesis and metastasis. The mechanism of this effect is poorly understood. In 2002 we reported that angiotensin, a powerful inhibitor of angiogenesis, binds to cell surface annexin II and inhibits angiogenesis. Annexin II is a well-established receptor for plasminogen and tPA that facilitates plasminogen activation to plasmin. We hypothesized that cell surface annexin II-dependent plasmin generation in tumor microenvironment regulates neoangiogenesis and breast tumor progression. To test this hypothesis we analyzed expression of tPA (tissue-type plasminogen activator) and annexin II in paraffin-embedded human breast cancer tissues. Surprisingly, we found an undetectable level of annexin in normal breast. By contrast, it was consistently expressed on the surface of invasive breast cancer and ductal carcinoma in situ (DCIS) indicating its involvement in breast cancer. To explore this in vivo data, we investigated annexin II and tPA expression by WB technique using a well-established invasive/metastatic MDA-MB231 cell line and the non-invasive/non-metastatic MCF-7 human breast cancer cell line. Our western blot analysis demonstrated selective expression of annexin II in MDA-MB231 cells but not in poorly invasive cells MCF-7 cells suggesting its potential role in breast cancer. Indeed, MDA-MB231 cells synthesized tPA interacts with cell surface annexin II. Since annexin II is a coreceptor for tPA and plasminogen, we tested whether MDA-MB231 cells are capable of producing plasmin in vitro. MDA-MB231 cells were incubated with recombinant tPA and kinetics of tPA was analyzed. Consistent with annexin II expression data only MDA-MB231 cells were capable of plasmin generation in a time-dependent manner while MCF-7 cells failed to convert plasminogen to plasmin. Since plasmin is known to induce neoangiogenesis, we analyzed a specific marker of neoangiogenesis (CD105) in breast cancer tissue. Consistently we found a correlation between annexin II expression and CD105 expression in human breast cancer tissues. These data strongly suggests that annexin II-dependent plasmin generation facilitates neoangiogenesis and breast cancer progression. These results suggest that annexin II may be an attractive target for new anti-angiogenic and anti-breast cancer therapies.

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P71-13: DIRECT PHYSICAL CONTINUATION OF NORMAL APPEARING BREAST TISSUES WITH MALIGNANT BREAST LESIONS

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The evolution of human breast cancer is believed to be a multistep process, progressing sequentially from normal to hyperplastic, to in situ, and to invasive stages. Progression from one stage to another is believed to be triggered by increasing accumulation of genetic abnormalities and expression of stage-restricted bio-molecules. Based on these theories, normal breast tissues and malignant breast lesions are supposedly to be segregated without direct physical continuation.

Our previous studies, however, have revealed that a subset of normal appearing breast tissues display a number of malignancy-associated alterations, including significantly elevated cell proliferation and genetic instabilities, the expression of malignancy-signature markers, and signs of direct stromal and vascular invasion. Together, our findings suggest a possibility of direct transformation from normal to malignancy. Our current study attempted to identify more direct evidence of such transformation.

Consecutive sections from human breast tumors (n=50) with co-existing normal, hyperplastic, in situ, and invasive components were subjected to double immunohistochemical staining and chromogenic in situ hybridization (CISH), to assess the protein expression and gene amplification of c-erbB2. The junctions between malignant and non-malignant cells were carefully examined to identify the direct physical continuation between normal and malignant tumor cells. The immunohistochemical and morphological profiles of different cell types at these junctions were compared with special attention to the cytological details.

A subset of morphologically normal appearing ductal cells were in direct physical continuation with their malignant counterparts. These normal appearing ducts consisted of a single layer of epithelial cells that were uniform in size and shape, with a normal nuclear and cytoplasmic ration and distinct polarity. These cells were completely devoid of c-erbB2 expression and overlaid non-disrupted ME cell layers. In sharp contrast, all the adjacent cancer cells appeared to be "budding" from focal ME cell layer disruptions, and were "puncturing" deep into the stroma. All the cancer cells were strongly and uniformly immunoreactive to c-erbB2 antibody, and also had a high level of c-erbB2 amplification. All the cancer cells were morphologically and immunohistochemically similar with markedly enlarged nuclei and nucleoli, and no surrounding ME cell layers. In over 20 such junctions examined, no intermediate cell type was seen between the normal and cancer cells.

These findings suggest that these normal and tumors cells are derived from different stem cells, and that focal ME cell layer disruptions facilitate monoclonal proliferation of tumor progenitor or stem cells, which could directly progress to invasive or metastatic lesions.

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P71-14: THE ROLE OF LAMININ 5 IN CANCER PROGRESSION

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Breast cancer is the most common type of cancer in women. The lifetime risk of developing breast cancer for a female born in the United States is 1:8. Metastasis is the major cause of breast cancer lethality. To metastasize, cancer cells have to break through the basement membrane. Laminin 5 (Ln-5) is one of the basement membrane proteins, consisting of three chains, $\alpha 3$, $\beta 3$, and $\gamma 2$. Ln-5 $\gamma 2$ chain contains DIII domain, a functional EGFR ligand, which can be released by MMP processing. It has been suggested by our lab that DIII domain may facilitate cancer progression by preventing anoikis. What we noticed is that there are paradoxical data in regard to the role of Ln-5 in cancer progression. For example, both the increased and decreased expression levels of Ln-5 subchains are reported in the literature. The fact that $\gamma 2$ chain exists in two different forms (as a secreted monomer or as a part of the Ln-5 heterotrimer) leads us to hypothesize that those two forms may play different roles in cancer progression. We hypothesize that $\gamma 2$ monomer is a tumor promoter while Ln-5 heterotrimer is a tumor suppressor.

We decided to test our hypothesis in breast cancer cell lines (MCF10A and MCF10A derived cell lines) together with a bladder carcinoma cell line (804G), which secrete Ln-5 heterotrimer. We are testing our hypothesis in two steps: (1) whether $\gamma 2$ monomer is positively correlated with cancer stages and (2) whether knocking down Ln-5 $\gamma 2$ can promote tumor progression. We made progress in both steps.

1. To detect whether $\gamma 2$ chain exists as a monomer, we decided to check the ratio between Ln-5 $\beta 3$ and $\gamma 2$ ($\beta 3/\gamma 2$ ratio). The lower the ratio is, the more likely it is for $\gamma 2$ monomers to be secreted. For the preliminary screening in cell lines, we used real-time PCR (RT-PCR). So far, we compared $\beta 3/\gamma 2$ between human breast gland cell lines MCF10A (nontumorigenic) and MCF10A-CA1a (tumorigenic). Our preliminary analysis reveals that the $\beta 3/\gamma 2$ ratio of the CA1a cells is lower than the $\beta 3/\gamma 2$ ratio of the MCF10A cells, which indicates that MCF10A-CA1a may produce more $\gamma 2$ monomer compared with MCF10A.

RT-PCR showed that MCF10A-CA1a (tumorigenic) has more $\gamma 2$ monomer than MCF10A (nontumorigenic). These data support our hypothesis that Ln-5 $\gamma 2$ monomer is tumor promoter.

2. We found that downregulation of Ln-5 $\gamma 2$ by shRNA in 804G cells, which synthesize Ln-5 heterotrimer, makes 804G cells more tumorigenic. E-cad stainings on tumor sections and cells cultured in vitro suggest that loss of $\gamma 2$ chain in the context of Ln-5 heterotrimer leads to loss of E-cad at cell-cell junction.

MCF10A Ln-5 $\gamma 2$ knockdown cells are generated as well. Cell-cell adhesion and animal studies in MCF10A $\gamma 2$ kd cells are in progress.

In summary, through this study, we may be able to establish $\gamma 2$ monomer as a marker for invasive breast cancer and Ln-5 heterotrimer as a tumor suppressor. It may also be established as a target for new breast cancer treatment development.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0308.

P71-15: THE ROLE OF THE TYPE III TGF- β RECEPTOR CYTOPLASMIC DOMAIN IN BREAST CANCER PROGRESSION

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The TGF β signaling pathway has a dichotomous role in breast cancer progression, acting in a tumor suppressive fashion early on, and switching to a tumor-promoting role in later stages. We have shown in vivo and in vitro that the type III TGF β receptor (T β RIII), long considered to act primarily as a co-receptor, directly mediates the suppression of breast cancer progression. Functionally, the cytoplasmic domain of T β RIII has been shown to be important in modulating the receptor's surface expression and downstream presentation of ligand.

To elucidate the mechanism by which T β RIII functions in breast cancer progression, we examined the contribution of the cytoplasmic domain. Our approach has been to examine its functional and mechanistic effects using both in vivo and in vitro strategies. In vivo, we used a murine mammary tumor line 4T1 that is syngeneic to Balb/c mice; this line was stably cotransfected with various T β RIII constructs and with the bioluminescent marker luciferase. After injection, primary and metastatic tumor growth were tracked non-invasively. In vitro, to demonstrate the contribution of the cytoplasmic domain in migration and invasion, functional assays were used; these included a scratch wound assay, transwell migration through fibronectin-coated wells, and the use of Matrigel transwell assays to measure invasive properties. Western blotting, along with binding and crosslinking of radioactively labeled ligand to receptors, were used to demonstrate protein expression and effects of downstream signaling mediators.

We demonstrate in vivo that the cytoplasmic domain is critical for regulating metastatic potential. Specifically, while full-length T β RIII suppressed metastatic growth and invasiveness, the T β RIII cytoplasmic deletion mutant had a phenotype similar to the T β RIII-deficient line; deletion of the cytoplasmic domain abrogated the ability of T β RIII to suppress metastasis. In vitro, we observe that the cytoplasmic domain has functional effects specifically affecting migration. Corresponding to the in vivo findings, full-length T β RIII suppresses migration while the T β RIII cytoplasmic deletion mutant failed to suppress migration. Finally, these effects are associated with changes in both Smad- dependent and Smad-independent signaling as evidenced by changes in the level of activation of downstream signaling mediators. In sum, these findings suggest a functional role for the T β RIII cytoplasmic domain in breast cancer progression.

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P71-16: GPR54 HAPLOINSUFFICIENCY DELAYS MMTV-PYMT-PREDISPOSED MAMMARY TUMOR PROGRESSION

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The G-protein coupled receptor GPR54 is implicated in the reproductive biological system in mammals, as there are some defects of organs for the reproductive system in GPR54 knockout mouse model system. Likewise, the formation of mammary ducts is deleted in GPR54 null mutant. Otherwise, GPR54 is also important for the disease states such as cancers, as Kisspeptin (an endogenous ligand of GPR54)-activated GPR54 can suppress the cancer metastasis. To understand the linkage between the mammary tumor progression and mammary gland development, we applied GPR54 heterozygous transgenic mouse model system expressing mammary tumor virus promoter-driven polyoma virus middle T antigen (MMTV-PyMT). Here, we demonstrate that GPR54 heterozygote shows the haploinsufficiency in the mammary gland development, which indicates GPR54 genocopy is relatively linked to phenotype. The number of either mammary ducts or mammary tumor masses at the terminal end buds is reduced in MMTV-PyMT-GPR54 mouse, compared with MMTV-PyMT. The expression level of markers for the mammary tumor progression such as cyclin D1, c-myc, or estrogen receptor alpha in tumors of MMTV-PyMT-GPR54 mice shows the delayed mammary tumor progression. Taken together, our data indicate the mammary tumor induction is tightly linked to the differentiation of mammary epithelia, suggesting GPR54 is important for the regulation of mammary gland development that leads to the mammary tumor predisposition.

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CELL MIGRATION/ INVASION II

Poster Session P72

P72-1: TOLL-LIKE RECEPTOR-9 AGONISTS STIMULATE BREAST CANCER INVASION

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Background: Toll-like receptor 9 (TLR9) is a member of the innate immune system that recognizes microbial and vertebral DNA. It is expressed intracellularly in the endosomal/lysosomal compartment. Stimulation of TLR9 by its agonists evokes an immune reaction including the production of pro-inflammatory cytokines. Intracellular signaling mediators downstream of TLR9 include MyD88 and TRAF6. In addition to mediating immunity, Toll, the *Drosophila* analog of mammalian TLRs, mediates also dorso-ventral patterning during embryogenesis. We showed previously that treatment with TLR9 agonistic, CpG-sequence containing oligonucleotides (CpG-oligonucleotides) induces MMP-13-mediated invasion in TLR9-expressing human breast cancer cell lines. The aim of this study was to further characterize the role of the TLR9 pathway in this process.

Methods: Human MDA-MB-231 breast cancer cells were stably transfected with a dominant-negative TRAF6 or a control cDNA. Peritoneal macrophages were isolated from wild-type, TLR9 -/- or MyD88 -/- mice. CpG-oligonucleotides sequence modifications were designed with the molecular modeling (MFOLD) software. Cellular invasion was studied with Matrigel assays in vitro. TLR9 expression in clinical breast cancer specimens was studied with immunohistochemistry.

Results: CpG-oligonucleotides (TLR9 agonists) induce invasion in macrophages from wild-type C57/B6 and MyD88 knockout mice. This effect is significantly inhibited in macrophages from TLR9 knockout mice and in human MDA-MB-231 breast cancer cells stably expressing dominant-negative TRAF6. CpG-oligonucleotide sequence modifications that resulted in changes in the stem-loop secondary structure influenced the invasion-inducing effect in MDA-MB-231 cells while methylation of the cytosine residues in the parent CpG-oligonucleotide did not. Expression of TLR9 was studied in clinical breast cancer samples and normal breast epithelium with immunohistochemistry. TLR9 staining localized in epithelial cells in both cancer and normal samples. The mean epithelial TLR9 staining intensity was significantly increased in the breast cancer specimens as compared with normal breast epithelial cells. Using another breast cancer cohort consisting of 119 patients, we compared TLR9 staining intensity in the primary tumors to other biological parameters. TLR9 staining was detected in < 97% of the specimens. TLR9 staining intensity was significantly higher ($p < 0.01$) in the estrogen receptor-negative (ER-) tumors, as compared with highly ER positive (ER++) tumors. TLR9 staining intensity was independent of progesterone receptor and Her2/neu expression and histological subtype of the tumor.

Conclusions and Potential Impact: TLR9 is frequently expressed in breast cancer. TLR9-agonist induced cellular invasion is mediated via TLR9 and TRAF6, independent of MyD88. The structure and/or stability of DNA may influence the induction of TLR9-mediated invasion in breast cancer. TLR9-mediated invasion may represent a novel pathway of breast cancer invasion, which may be significant especially in ER-breast tumors. Further studies are needed to identify physiological TLR9 agonists that may activate this invasive pathway in breast cancer. If TLR9 proves significant in the pathogenesis of breast cancer then the efficacy of TLR9 inhibitors, such as chloroquine, should be tested in breast cancer patients.

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P72-2: AN IN VITRO STUDY OF BREAST CANCER INVASION INTO THE LYMPHATICS

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Background and Objectives: Lymphatic metastasis is a major prognostic factor for breast cancer patients, but the mechanisms underlying lymphatic dissemination are poorly understood. Actively growing tumors have leaky blood vessels and high interstitial fluid pressure; hence, fluid exudes from the tumor into surrounding stroma where it is collected by lymphatics. Furthermore, chemokine receptor CCR7 has been correlated with poor patient prognosis, but the significance of this observation is unclear. We hypothesized that this biophysical environment, along with the resulting transport of tumor-secreted CCR7 ligand, act together to help tumor cells "find" functional lymphatics and escape to form metastases.

Methodologies: To investigate this hypothesis, we developed an in vitro coculture model to recreate the biophysical environment experienced by a tumor. Such a tissue-engineered system would require multiple aspects of the tumor-extracellular matrix-

lymphatic including tumor cells, 3D matrix, and lymphatic endothelial cells to which we could add low levels of interstitial fluid flow (within physiological range) as would occur in vivo.

Results: This model was used to identify a novel mechanism of tumor cell homing to lymphatics that we termed "autologous chemotaxis." We observed that breast cancer cells themselves (but not normal mammary epithelial cells) were able to produce CCR7 ligand and that secretion was significantly enhanced when cells were maintained in a relevant 3D matrix compared with 2D culture, indicating the importance of the micro-environment when studying tumor cell behavior in vitro. Tumor cells, but not normal cells, also migrated to lymphatic endothelial cells—a response that was prevented when CCR7 signaling was blocked. Moreover, in the absence of lymphatics, tumor cell-secreted chemokine in the presence of flow alone could promote autocrine chemotaxis in the direction of flow (toward draining lymphatics); this response was entirely CCR7 dependent but also LEC independent further pointing to a flow-enhanced autocrine signaling mechanism in invasion.

Conclusions: In summary, we show how breast cancer cells use interstitial flow to create and amplify autologously generated chemokine gradients and thus chemotact toward local lymphatics simply by virtue of their normal draining function when too far away to detect putative lymphatic-derived signals. This work reveals the first evidence of autologous chemotaxis and presents a novel mechanism that explains how tumor cells may be "guided" toward lymphatics during early metastasis and potentially identifies viable targets for future anti-invasion/metastasis therapies.

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P72-3: MICRORNA-21 REGULATES TUMOR GROWTH AND INVASION BY TARGETING MULTIPLE TUMOR SUPPRESSOR GENES

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Background: MicroRNAs (miRNAs) are a class of small noncoding RNAs that control gene expression by targeting mRNAs and triggering either translation repression or RNA degradation. Mounting evidence suggests that miRNAs may function as oncogenes or tumor suppressor genes and play a key role in tumorigenesis. The objective of this study is to determine the role of microRNA-21 (*mir-21*) in breast cancer because *mir-21* was overexpressed in breast tumor compared to the matched normal breast tissue.

Methods: We used TaqMan real-time PCR method to profile miRNA expression in matched breast tumor specimens. Since *mir-21* was found to be overexpressed in the breast tumors, we used antisense oligonucleotide approach to knock down *mir-21* and to determine the role of *mir-21* in breast tumorigenesis. Therefore, breast tumor cell lines MCF-7 and MDA-MB-231 were transfected with either negative control oligonucleotide or anti-*mir-21* oligonucleotide (anti-*mir-21*) and then injected into nude mice. The primary tumor and lung metastasis were determined. To determine the underlying mechanism of *mir-21*-mediated tumor growth and invasion, we searched for *mir-21* target genes by proteomic and a genetic selection method combined with in silico prediction methods.

Results: We demonstrated that *mir-21* affected not only tumor growth but also cell invasion and metastasis. In MCF-7 cells, we found that suppression of *mir-21* by anti-*mir-21* substantially inhibited cell growth in vitro and tumor growth in a xenograft breast carcinoma mouse model. However, in the metastatic MDA-MB-231 cells, suppression of *mir-21* had no significant effect on in vitro cell growth, but it suppressed invasion and metastasis significantly. To determine the underlying mechanism of *mir-21*-mediated tumor growth and invasion, we attempted to identify potential *mir-21* target genes by proteomic and a genetic selection method along with in silico prediction methods. We demonstrated that tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), and maspin were direct targets for *mir-21*. Further study indicated that *mir-21* targeted these three genes by interacting with a putative *mir-21* binding site at the 3'-untranslated region (3'-UTR). Of particular interest, we found that expression of PDCD4 and maspin inversely correlated with *mir-21* expression in human breast tumor specimens, highlighting the clinical relevance of potential *mir-21* regulation of PDCD4 and maspin.

Conclusion: Taken together, our results suggest that, as an oncogenic miRNA, *mir-21* can suppress multiple tumor suppressor genes involved in tumor growth, invasion, and metastasis. Therefore, targeting *mir-21* may provide a novel approach for treatment of advanced breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0471 and National Cancer Institute.

P72-4: FIBRINOGEN INDUCES ENDOTHELIAL CELL PERMEABILITY AND PROMOTES TRANSENDOTHELIAL MIGRATION OF MDA-MB-231 BREAST CANCER CELLS

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Background and Objective: Inflammatory breast cancer (IBC) is a highly aggressive form of primary and metastatic breast cancer and is associated with a high incidence of metastasis. IBC is also the most lethal form of locally advanced breast cancer. Systemic inflammation is clearly linked with adverse prognosis in patients with cancer and is characterized by elevated expression of pro-inflammatory mediators, including the blood-clotting protein fibrinogen (Fg). Fg is a key component of tumor stroma-extracellular matrix and promotes tumor cell metastasis, but the molecular mechanisms by which this occurs are poorly understood. A portion of the Fg beta-chain (Fg β 15-42), which binds to vascular endothelial cell (EC) adhesion molecule VE-cadherin, promotes cellular responses of adhesion, proliferation, and migration during angiogenesis and tumor growth. The present study sought to determine whether Fg β 15-42 sequences promote cancer cell invasion through an EC barrier, an essential step in metastasis.

Methods: The effects of Fg on modulating EC permeability to FITC-dextran and migration of highly invasive MDA-MB-231 breast cancer cells (231) across an EC barrier were measured using a standard Transwell insert culture system. Vascular endothelial growth factor (VEGF) was the positive control. Nonmalignant breast MCF-10A cells served as a negative cell line for permeability studies. Antibodies that bind to Fg β 15-42 (T2G1), fibrinopeptide B (18C6), and VE-cadherin (BV9) were used to test whether Fg β 15-42 plays a role in induction of EC permeability. Immunofluorescent assays (IFA) were used to monitor cell migration.

Results: Plasma Fg dose-dependently induced a 200,000-fold increase in flux of FITC-dextran across an apical EC monolayer (akin to a blood vessel lumen) to the bottom chamber (representative of tissue stroma) in 15 min, peaking between 45–60 min. VEGF induced a 150,000-fold increase in FITC-dextran flux in 15 min. IFA confirmed that Fg induced gaps between EC and that VE-cadherin relocated from cell membranes to the cytoplasm. In contrast, Fg did not induce MCF-10A cell permeability, indicating that Fg-induced permeability is cell-type-specific. Both T2G1 and BV9 but not 18C6 partially inhibited Fg-induced FITC-dextran flux, implicating VE-cadherin and Fg β 15-42 in mechanisms of Fg-induced EC permeability. Furthermore, both VEGF (positive control) and Fg significantly enhanced migration of 231 cells across quiescent EC over that observed for untreated cells. Confocal microscopy confirmed transmigration and adherence of breast cells to the bottom side of the insert.

Conclusions: The data in this report indicate that Fg β 15-42 sequences play a significant role in inducing permeability of an EC barrier in part by disrupting VE-cadherin-dependent cell-to-cell adhesion. Furthermore, the ability of highly invasive breast cancer cells to transmigrate from the apical to the basal side of an EC barrier (akin to extravasation of cancer cells) was significantly enhanced in the presence of Fg. Hence, Fg β 15-42 sequences represent a molecular target for therapeutic intervention of cancer cell metastasis, and possibly tumor-associated angiogenesis, by disrupting cell-to-cell contacts. Identifying molecular targets for therapeutic intervention of breast cancer cell metastasis, recruitment of inflammatory cells, and angiogenesis will contribute to the reduction of the morbidity and mortality associated with breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0603.

P72-5: THE TUMORIGENIC POTENTIAL OF RECONSTITUTED TISSUES WITH PREDEFINED CELLULAR HISTOLOGY; A MODEL FOR TUMOR INVASION

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Genetic or Epigenetic Stromal Changes in Epithelial Cancer Etiology: Cancer progression is thought to be the product of accumulated mutations in cancer cells. Yet, inherent properties of the cancers neighboring cells, collectively termed stroma, have recently been implemented in disease progression using mouse-xenograft models. What drives this stromal-induced phenotype? The genomic integrity of stroma is a focus of controversy, but the differences in gene expression in these cells are generally agreed upon. We have observed that expression levels of many genes are changed in cancer associated fibroblasts as a direct consequence of signal transduction invoked by the neighboring epithelial cell. This suggests a direct role of stromal-epithelial crosstalk in the xenograft outcome.

The Technical Approach to Reconstituting Tumor Microenvironment: To address the etiological role of the invasion event in cancer, in terms of cell-cell communication, xenografts were formed with defined tissue arrangements, either an epithelial sheath on stroma with separating basement membrane or intermixed collagen stabs. First we embedded cells in collagen and Matrigel[®] to custom-design tissue architectures ("malignant"/intermixed or "normal"/layered) and transplanted those organotypic cul-

tures in immune compromised mice and followed the tumor-burden of the emerging xenografts. We applied epithelial and fibroblast cells from defined steps in cancer progression.

Findings: We found that CAFs promote breast epithelial cancer growth independent of the cellular histology and configuration. By contrast, 1[°]Fs eliminated cancer growth only in the normal cellular histology. Immunohistochemistry shows that the xenografts all divide in the same rate (Ki67), but the 1[°]Fs induce cell death (activated Caspase 3) in the cancer cells. This increase of cell death is restricted to the fibroblast-cancer cell interface only when the two cell populations are in the "normal"/layered arrangement. We used cDNA microarrays to profile and dissect intercellular signaling that differs between those artificial tissues. We can observe cell death specifically with the 1[°]Fs using cell imaging systems in vitro. We also observe a robust and specific chemoattraction of the cancer cells to the CAFs. Microarray comparison of gene expression in those artificial tissues implies candidate genes as mediators of this restraint. A configuration-independent reaction between the normal fibroblast and the cancer cell appears to be driven by TNF α . We show that this inflammatory reaction is a highly frequent feature in multiple cancers using microarray profiles.

Significance: This is compelling evidence to suggest that neighboring cells restrain genetically transformed cells in a context-dependent manner and that disruption of tissue morphology releases sporadic transformed cells from such restraint. We now aim to inhibit the migration toward CAFs and enhance the 1[°]F-mediated cell killing as potential future therapeutics.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0720.

P72-6: HUMAN BREAST MYOEPIITHELIAL CELLS CAN EXHIBIT LYMPHOVASCULOGENIC MIMICRY

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Ohio State University

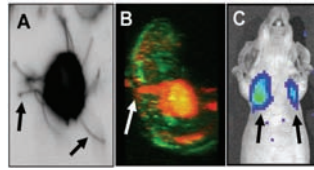
Our previous studies had indicated that human myoepithelial cells exert tumor suppressive effects on ductal carcinomas in situ (DCIS) including antiinvasive, antiproliferative, and antiangiogenic effects. More recently others have demonstrated that myoepithelial cells, in turn, can be paracrinally regulated by DCIS and undergo alterations in gene expression and promoter methylation. In these latter studies, findings suggested that myoepithelial cells can become less differentiated and express endothelial-related genes. Recent evidence generated by others has also suggested that experimental carcinomas may short-circuit the process of angiogenesis by inducing vasculogenic mimicry, whereby tumor cells either directly differentiate or indirectly stimulate stem cells to differentiate into lymphovascular channels. Because vasculogenic mimicry has been observed in only a few experimental model systems and even fewer human cancers, we wondered whether we might find direct evidence for this phenomenon in human breast carcinomas and myoepithelial cells both experimentally as well as observationally. Experimentally we used a human xenograft model of inflammatory breast cancer (MARY-X), a model that exhibited a DCIS pattern centrally and lymphovascular invasion (LVI) peripherally to generate tumoral spheroids in vitro. Unlabelled MARY-X tumoral spheroids co-injected with either GFP or RFP-labeled human myoepithelial cells (HMS-1) or murine embryonal fibroblasts (MEFs) into nude or GFP-transgenic nude mice gave rise to tumors that exhibited GFP or RFP fluorescence. The appropriately colored fluorescence was present within the emerging tumor nodules in both the nude and GFP-transgenic nudes. Interestingly in the experiments involving the mixture of unlabelled spheroids and RFP-labeled HMS-1 or MEFs in GFP-transgenics, the tumor nodules initially exhibited a red fluorescence that over time gave rise to a hybrid yellow fluorescence. On IHC examination of the extirpated tumor nodules, circumferential GFP or RFP immunoreactivity was observed surrounding the tumor cell clusters. On closer examination many of these clusters were within lymphovascular spaces that contained luminal erythrocytes and that exhibited GFP/RFP immunoreactivity within their lining endothelium. In concurrent observational studies, 10 cases of breast DCIS, where areas of LVI were observed adjacent to areas of DCIS, were studied by morphometry as well as by IHC with tumor proliferation (Ki-67) and adhesion (E-cadherin) markers, myoepithelial (p63), as well as lymphovascular (D2-40 and CD31) markers. The DCIS clusters exhibited identical proliferation, E-cadherin immunoreactivity and size (perimeter) as the tumor emboli within lymphatic channels ($p = .5$), suggesting that these structures were one and the same and remained intact during their DCIS to LVI transition. The DCIS clusters exhibited a progressive loss (50%–100%) of p63 myoepithelial immunoreactivity. The tumor emboli were exclusively present within lymphatic channels whose dual p63/CD31 and p63/D2-40 immunoreactivities and their relatively weak pattern of D2-40/CD31 staining suggested that they represented immature and newly created vasculature derived from myoepithelial-lined ducts. The experimental as well as observational studies suggested that human breast carcinomas can progress to LVI by inducing lymphovasculo-genic mimicry from either myoepithelial or mesenchymal stem cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0176; California Breast Cancer Research Program; and Susan G. Komen for the Cure.

P72-7: MICROTENTACLES FORMED FROM KINESIN-DEPENDENT COORDINATION OF VIMENTIN AND DETYROSINATED TUBULIN PROMOTE THE ADHESION OF DETACHED BREAST TUMOR CELLS

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Successful attachment of circulating breast tumor cells in distant tissues is an important determinant of metastatic efficiency. Our current research reveals that detached human breast tumor cell lines produce novel protrusions of the plasma membrane that promote the reattachment of tumor cells. We have termed these protrusions "microtentacles" due to clear mechanistic distinctions from either actin-based filopodia/invadopodia or tubulin-based cilia. Time-lapse video microscopy indicates that the rapid extension and dynamic motion of microtentacles relies on kinesin-dependent extension of vimentin along stabilized microtubules that are enriched in detyrosinated tubulin. Previous studies indicate that elevated levels of vimentin and detyrosinated tubulin in tumors predict poor patient outcome although the specific mechanisms remain unclear. In addition, compounds long known to inhibit the metastatic efficiency of circulating tumor cells have recently been shown to target kinesin motor protein activity. We propose that the independent roles of vimentin, detyrosinated tubulin, and kinesin in tumor metastasis could result from their interdependent contributions to microtentacle formation. Microtentacles are clearly antagonized by the actin cytoskeleton, unlike invadopodia and filopodia, which depend on actin filaments. Human breast tumor cell lines that display a disorganized actin cortex and hallmarks of epithelial-to-mesenchymal transition produce higher frequencies and greater extension of microtentacles. Recent studies using intravital microscopy indicate that a mechanism consistent with microtentacles is responsible for the attachment of circulating tumor cells to blood vessel walls. We are currently using time-lapse confocal microscopy of tumor cells circulating in intact blood vessels and whole-animal optical imaging to define the role of these unique microtentacles during breast tumor metastasis.



Imaging how microtentacles(A) affect circulating tumor cell binding to vessel walls(B) and lungs(C).

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0383.

P72-8: A GENOME-WIDE RNA INTERFERENCE SCREEN IN HUMAN MAMMARY EPITHELIAL CELLS TO IDENTIFY GENES INVOLVED IN ANOIKIS

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Normal endothelial and epithelial cells are dependent upon interactions with the extracellular cell matrix (ECM) for survival and proliferation; when they lose contact with the ECM, they undergo a specialized form of programmed cell death termed anoikis. By contrast, cancer cells are able to survive and grow in the absence of anchorage to the ECM—a hallmark of many different types of human cancers, including breast cancer. The acquisition of anoikis resistance is a critical step during tumor development and metastasis and, importantly, has been shown to contribute prominently to the malignancy of breast cancer. Genes that induce anoikis (i.e., anoikis effector genes) in mammary epithelial cells represent potential new targets for breast cancer therapy. Thus, a better understanding of the molecular mechanisms underlying anoikis will likely open new avenues for the treatment of breast cancer.

The objective of our study is to identify genes that: (1) mediate anoikis in human mammary epithelial cells and (2) when downregulated, confer tumorigenesis or metastatic potential. Toward this goal, we have performed a genome-wide RNA interference-based screen to identify genes that, when knocked down, protect human mammary epithelial (MCF10A) cells from anoikis. MCF10A cells are first treated with a human short hairpin RNA (shRNA) library and then plated on poly-hydroxyethylmethacrylate (HEMA)-coated plates; under these conditions, cells cannot attach to the plate surface and are forced to grow in suspension (i.e., anchorage-independent growth). Our initial attempt at the screen was hampered by the fact that although the majority (>90%) of cells undergo apoptosis, the small "false-positive" background population of surviving cells complicates the identification of true-positives. To circumvent this problem, we have combined the genome-wide shRNA screening strategy with massively parallel signature sequencing (MPSS), a technology that enables the quantitative comparison of nucleic acid profiles. Using this approach, we compare the quantitative profiles of the initial shRNA population (prior to growth in suspension) to that following growth in suspension. An shRNA that knocks down an anoikis effector gene (a true-positive) is defined as one whose relative representation is significantly increased following growth in suspension.

Positives from this screen will be followed up by cancer profiling database mining to determine whether the anoikis effector genes are dysregulated in breast cancer. Candidate genes will be further tested in tumorigenicity and spontaneous lung micrometastasis assays using a xenograft mouse model to determine whether downregulation of any of the anoikis effector genes confers tumorigenicity and/or metastatic potential upon normal mammary epithelial cells. By combining a functional genome-wide screening/MPSS approach with expression profiling and in vivo studies, we aim to generate a comprehensive list of genes that play a physiological role in breast cancer metastasis. Identifying genes that regulate metastatic growth represents a key first step in developing useful tools for diagnosis and therapeutic strategies that specifically delay or inhibit metastasis of invasive breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0381.

P72-9: HUMAN FETUIN-A IS A STRONG PROMOTER OF ANCHORAGE-INDEPENDENT GROWTH AND SURVIVAL OF BREAST CARCINOMA CELLS

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 Meharry Medical College, Nashville

Fetuin-A, a major component of fetal bovine serum used in growth medium as well as a major serum protein synthesized by the liver, has been established to prevent ectopic calcification in vivo. We on the other hand, have demonstrated that fetuin-A (ahsg) is a growth promoter of breast carcinoma cells in vitro. Experiments are currently ongoing in our laboratory to ascertain whether it also influences the initiation, promotion, and/or progression of breast cancer using the PymT transgenic mouse model for breast cancer. The most current studies in our laboratory now point to its potential as a powerful regulator of growth of breast carcinoma cells in vitro. We hypothesized in the present studies that one of the main functions of human fetuin-A (ahsg) is to modulate apoptotic cell death and to promote anchorage-independent growth in breast carcinoma cells. Breast carcinoma cells (BT-549, MDA-MB-231, MDA-MB-435, and MCF-10A) were maintained in DMEM/F12 containing 10% heat inactivated fetal bovine serum. The cells were then dislodged from culture dishes with 2 mM EDTA and washed twice in serum-free medium. They were then seeded at 5,000 cells/well in 96-well microtiter plates in the absence or presence of graded doses of human fetuin-A (ahsg) or another serum glycoprotein with similar physico-chemical properties and anchorage-independent growth (assessed as colonies loosely attached to the wells) evaluated. These data demonstrate that in the presence of ahsg concentrations as low as 50 µg/mL, the cells grow as large clumps while in the presence of equivalent concentrations of irrelevant proteins, this anchorage growth capacity was highly reduced. The cells also remain alive much longer in the presence of ahsg compared to the irrelevant proteins. The growth is evident as early as 1-week post-plating. We postulate that ahsg signals the growth and abrogates apoptotic cell death via the PI3 kinase/Akt pathway. An alternative mechanism may involve the alteration of extra-cellular calcium sensing in the presence of ahsg. In conclusion, it is becoming clear that fetuin-A in vivo may protect breast tumor cells (possibly tumor stem cells) from apoptotic cell death and enhance their anchorage-independent growth, as well as tumor emboli formation, which leads to enhanced metastatic spread of the disease.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0254.

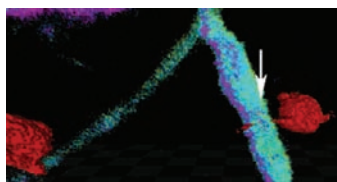
P72-10: ACTIVATION OF MYOSIN LIGHT CHAIN KINASE IN ENDOTHELIUM BY INVADING BREAST CANCER CELL: A 3D FRET STUDY

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 Northwestern University

To investigate the signaling events involved in tumor metastasis, we have devised a three-dimensional matrix system that allows endothelial cells to form vasculature network in vitro. This vasculature network is engineered using endothelial cells stably expressing a FRET biosensor for myosin light chain kinase (MLCK) activity (*J. Cell Biol.* 156: 543-553, 2002), thus integrating FRET biosensor microscopy and tissue engineering to create a dynamic 3D assay wherein tumor transendothelial migration can be studied with very high spatial and temporal resolution from the perspective of the endothelium.

The in vitro 3D vasculature network exhibits lumen formation as characterized both by confocal as well as transmission electron microscopy. In situ immunostaining shows that the endothelial cells deposited α4 laminin on the peripheral area of the vessel tube but the luminal space is relatively cleared of synthesized α4 laminin, indicating that the endothelial cells that form the in vitro vasculature undergo polarization, as would normal endothelium under physiological condition. Upon the addition of MDA-MB 231 breast cancer cells pre-labeled with CellTracker Red, we frequently observed the invadopodia of the breast cancer cell protruding into the cytoplasm of the endothelial cells, rather than through the cell junction as expected. The cytoplasm of the two cells can be

easily delineated by confocal microscopy as the endothelial cell and the tumor cell are labeled with different colors. It is also common to observe entire cancer cells enveloped within the lumen of the endothelial cell. This is consistent with the recently observed transcellular migration of peripheral blood mononuclear cells and MCF cancer cells (*Nature Cell Biol.* 8: 156-161, 2006; *Cell* 131:966-979, 2007), in that invading cells do not always penetrate the endothelial layer through the cell-cell junction, but rather by directly compromising the integrity of the endothelial cell membrane. Using our MLCK FRET biosensor, we show that MDA-MB 231 cancer cells induce transient and regional MLCK activation within the endothelial cells at the site of tumor-endothelium contact—suggesting that the endothelial myosin II activity may be locally affected as the cancer invadopodia protrude into the endothelial cells.



Breast cancer cell invadopodia (red) penetrating an endothelial vessel tube (arrow) expressing myosin light chain kinase FRET biosensor

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0345.

P72-11: CONTROL OF PROLIFERATION, MIGRATION, AND INVASION OF RAT BREAST TUMOR CELLS BY PK11195, AN ANTAGONIST OF PERIPHERAL BENZODIAZEPINE RECEPTORS (PBRs)

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Although some rat breast cancer epithelial cell lines have been reported in the literature, only a few are currently available for research, which limits the number of studies that can be performed. This study reports the isolation, establishment, and characterization of rat breast tumor epithelial cells, and the effects of a PBR antagonist on these cells. Breast tumors were induced by administration of a carcinogen, dimethylbenz[*a*]anthracene (DMBA) to 50-day old female rats maintained on a standard AIN-76A diet with casein as the protein source. The tumors were developed approximately after 120 days. The tumors were of grade I (20%), grade II (60%), and grade III (20%). The grade III tumors were cultured in DMEM/F12 media with supplements. The maintenance of primary rat breast epithelial cells is a difficult task. However, we were successful to grow these cells up to 30 passages for cellular characterization.

These cells were highly proliferative (measured by WST-1 assay), invasive (measured by matrigel invasion assay), angiogenic (measured by immunohistochemistry with CD-31 and VEGF antibodies), and possessed migrating ability/motility (measured by circular wound healing assay and quantitated by Metamorph and Image J software). Furthermore, these cells expressed elevated levels of peripheral benzodiazepine receptor (PBR), a cancer promoting gene (measured by immunohistochemistry and immunofluorescence with PBR antibodies). The proliferation, invasion and migration appear to

decrease when treated with PK11195, a PBR antagonist. Thus, PBR antagonist may be a potential therapeutic agent for the control of breast cancer aggressiveness. Currently, we are trying to immortalize these cells for long-term use.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0352 and Flight Attendant Medical Research Institute.

P72-12: AUTOCRINE MIGRATION-PROMOTING ROLE OF VASCULAR ENDOTHELIAL FACTOR-C IN CYCLOOXYGENASE-2 EXPRESSING HUMAN BREAST CANCER CELLS DUE TO BINDING OF ENDOGENOUS VEGF-C TO VEGF-C RECEPTORS

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University of Western Ontario

Background and Objectives: Overexpression of VEGF-C, a lymphangiogenic factor, is associated with increased lymph node metastasis and poor prognosis in human breast cancer. We have shown that VEGF-C is upregulated in human breast cancer by cyclooxygenase(COX)-2, at least in part due to activation of EP1 and EP4 receptors by endogenous PGE₂. While both COX-2 and VEGF-C expression in breast cancer tissues was positively associated with the expression of LYVE-1, a marker for lymphatic endothelium, we found no significant difference in the levels of immunodetectable VEGF-C protein in tissues with high versus low lymphovascular density, suggesting additional lymphangiogenesis-independent role(s) of VEGF-C. We tested the hypothesis that tumor-derived VEGF-C may play an autocrine role in metastasis by promoting cellular motility through one or more VEGF-C-binding receptors VEGFR-2, VEGFR-3, neuropilin (NRP)-1, NRP-2, and integrin α 9 β 1.

Methods and Results: We investigated the expression of these receptors (at the mRNA or protein levels) in several breast cancer cell lines (MDA-MB-231, Hs578T, SK-BR-3, T-47D, and MCF7) and their possible requirement in migration of two VEGF-C-secreting, highly metastatic lines MDA-MB-231 and Hs578T. While cell lines varied significantly in their expression of above VEGF-C receptors, migratory activity of MDA-MB-231 and Hs578T cells was linked to one or more of these receptors. Depletion of endogenous VEGF-C by treatments with a neutralizing antibody, VEGF-C siRNA or inhibitors of Src, EGFR/Her2/neu, and p38 MAP kinases that inhibited VEGF-C production, inhibited cellular migration, indicating the requirement of VEGF-C for migratory function. Migration was differentially attenuated by blocking or down-regulation of different VEGF-C receptors, e.g., treatment with a VEGFR-2 tyrosine kinase inhibitor, NRP-1 and NRP-2 siRNA or α 9 β 1 integrin antibody, indicating the participation of one or more of the receptors in cell motility.

Conclusions: This novel migration promoting role of tumor-derived VEGF-C associated with a diverse group of VEGF-C receptors indicates that breast cancer metastasis can be promoted by coordinated stimulation of lymphangiogenesis and enhanced migratory activity of breast cancer cells.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6096; Canadian Breast Cancer Foundation (Ontario Chapter); and Ontario Institute of Cancer Research.

METASTASIS II

Poster Session P73

P73-1: THE SUPPRESSION OF BREAST CANCER METASTASIS BY BONE MORPHOGENIC PROTEINS

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The majority of deaths due to breast cancer result from the formation of metastases that arise in distant organs such as lymph nodes, lung, liver, and bone. These secondary tumors result in organ failure, spinal cord compression, bone pain, and fractures. To date, there is no effective cure and current treatments are largely palliative.

To enhance our current understanding of the molecular events regulating metastasis, we have utilized a mouse model of spontaneous breast cancer metastasis to reveal these genetic determinants. We array profiled several tumor lines from this model and correlated gene expression with metastatic propensity in vivo. Gene ontology analysis revealed that highly metastatic tumors expressed a greater percentage of "cell communication" genes, an ontology populated with cell signaling and extracellular matrix (ECM) genes (1). Bone morphogenic protein-4 (BMP4), a cytokine involved in tissue patterning and morphogenesis, had reduced expression in highly metastatic tumors and was further characterized for its role in metastasis.

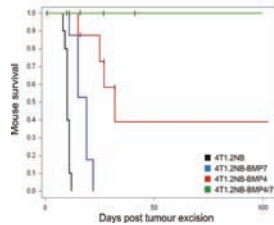


Figure 1. Survival following inoculation of 4T1.2 cells expressing either BMP4 or BMP7 or both

The overall survival of mice bearing 4T1.2 primary tumors engineered to express exogenous BMP4 (4T1.2-BMP4) was significantly greater than those bearing the parental 4T1.2 tumors. This was attributed to decreased tumor cell escape from the primary site and a marked reduction in detectable secondary tumors in most mice. Similar results were obtained in mice bearing 4T1.2 tumors expressing BMP7 or both BMP4 and BMP7, suggesting that BMPs can modulate the suppression of breast cancer metastasis (Figure 1). Gene expression profiling revealed that BMP4 induces expression of Id1, Id3, and SMAD7 in the 4T1.2 tumor cells. The role of these BMP-regulated genes in the metastatic process is under investigation.

The clinical efficacy of BMP4 as an antimetastatic therapy was also assessed by administration of recombinant BMP4 (rBMP4) to mice bearing 4T1.2 tumors. Mice receiving rBMP4 had a significant increase in overall survival compared to controls, suggesting that BMPs could be utilized as an adjuvant therapy for metastatic disease. Taken together, these results demonstrate a novel role for BMP4 and BMP7 as breast cancer metastasis suppressors.

1. Eckhardt et al., 2005. *Molecular Cancer Research* 3: 1-13.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-05-1-0442; National Health & Medical Research Council of Australia; and Susan G. Komen for the Cure.

P73-2: REPROGRAMMING METASTATIC BEHAVIOR WITH ARTIFICIAL TRANSCRIPTION FACTORS

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 University of North Carolina at Chapel Hill

Metastatic spread, and not primary tumor burden, is the leading cause of breast cancer deaths. The development of metastatic behavior during breast cancer progression is a dynamic process thought to require the concerted action of multiple genes. The transcriptional programs cooperatively required for the malignant progression of breast tumors are largely unknown. In addition, novel therapeutic strategies should be able to target multiple targets dysregulated during disease progression. In this project we propose the isolation of artificial transcription factors (ATFs) for the discovery of gene panels, which cooperate during the generation of metastatic behavior. An ATF is made by linkage of a DNA-binding domain (DBD) with a transcriptional effector domain, which mediates activation or repression of endogenous genes. ATFs are typically made of arrays of Cys2-His2 zinc finger (ZF) domains. Importantly, ATFs with these ZF domains can be linked to both activator and repressors of transcription, facilitating both up- and down-regulation of tumor cell phenotypes. We have developed a novel genome-wide approach for the functional identification and regulation of genes involved in tumor progression. We have generated libraries of ATFs by recombination of large repertoires of sequence-specific ZF domains. ZF domains were linked to an activator (VP64) or repressor (SKD) of transcription. When delivered into tumor cell populations, ATF libraries have the potential to activate or repress virtually any gene. The objective of this project is to apply ATF libraries to identify and regulate genes that cooperate during the process induction of breast cancer cell invasion and progression. We have delivered ATF libraries into noninvasive breast cancer cell lines. We have selected ATFs able to induce or enhance breast cell invasion. The ATF-selections were

performed in vitro using matrigel invasion assays. ATFs-expressing cells have been profiled using DNA microarrays to determine genes differentially regulated by the ATF that are responsible for the phenotype change. The regulation of these markers was further validated by real-time PCR and other analyses. The transcriptional profiles of ATF-expressing cells were compared with available microarray profiles of highly metastatic breast tumors. We expect this project will lead to the functional identification of novel markers of breast cancer disease progression that could be used as early predictors of malignant behavior. In the future, these ATFs could be used as master genetic switches to modulate malignant behavior in in vivo models of breast cancer.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0477.

P73-3: BREAST CARCINOMA CELLS IN THE METASTATIC ENVIRONMENT RE-EXPRESS E-CADHERIN AS A SURVIVAL MECHANISM

Alan Wells, Yvonne Chao, Michelle Echko, and Christopher Shepard
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Epithelial cadherin's (E-cadherin) transcriptional silencing in most advanced tumors, due to promoter methylation, enables tumor cells to disseminate from the primary mass. However, E-cadherin-positive metastatic carcinoma foci do originate from mainly E-cadherin-negative primaries. It is unknown if this is due to dissemination of a minor population of E-cadherin positive cells or re-expression of E-cadherin during metastasis. Here, we demonstrate that co-culture of invasive E-cadherin-negative breast cancer cells with hepatocytes, the second most common site of soft tissue metastasis for breast cancer, triggers an epigenetic reversion in breast cancer cells resulting in demethylation of the E-cadherin promoter and subsequent expression on the protein level. This demethylation was demonstrated by methylation-specific PCR amplification. Demethylation of the E-cadherin promoter is coupled to proliferation of the cancer cells and is not the result of a global demethylation program, as inhibition of proliferation prevented re-expression of E-cadherin. We show a similar time-course for E-cadherin upregulation in 3 of 11 of primary human breast cancer explants co-cultured with primary hepatocytes. Further, we show that E-cadherin ligation between breast cancer cells and hepatocytes is functional and activates the canonical MAPK and Akt pathways in these cancer cells. This E-cadherin binding makes the breast cancer cells more resistant to apoptotic signals (tumor necrosis factor- α) and chemotherapies (camptothecin). Our epigenetic-reversion hypothesis for E-cadherin represents not only a paradigm shift in the current thinking that absence of E-cadherin is fundamental for metastasis, but also potentially reveals mechanisms underlying the failure to readily treat the early stages of metastatic disease in breast cancer patients with existing therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0480 and Veterans Affairs.

P73-4: ANGIOPOIETIN-2 STIMULATES BREAST CANCER METASTASIS THROUGH THE $\alpha 5 \beta 1$ INTEGRIN-MEDIATED PATHWAY

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Acquisition of a metastatic phenotype by breast cancer cells includes alternations of multigenic programs that permit tumor cells to metastasize to distant organs. Here, we report that angiopoietin-2, a known growth factor, is capable of promoting breast cancer cell invasion leading to metastasis. Analysis of 185 primary human breast cancer specimens that include 97 tumors demonstrating lymph node and/or distant metastasis reveals a significant correlation between the expression of angiopoietin-2 and E-cadherin, Snail, metastatic potential, tumor grade and lymph-vascular invasion during breast cancer progression. Using a xenograft model, we show that overexpression of angiopoietin-2 in poorly metastatic MCF-7 breast cancer cells suppresses expression of E-cadherin, induces Snail expression and phosphorylation of Akt and glucocorticoid synthase kinase (GSK)-3 β promoting metastasis to the lymph nodes and lung. In cell culture, angiopoietin-2 promotes cell migration and invasion in Tie2-deficient breast cancer cells through the $\alpha 5 \beta 1$ integrin/ILK/Akt, GSK-3 β /Snail/E-cadherin signaling pathway. Inhibition of ILK and the $\alpha 5 \beta 1$ integrin abrogates angiopoietin-2 modulation of Akt, GSK-3 β , Snail and E-cadherin and angiopoietin-2-stimulated breast cancer cell migration and invasion. Together, these results underscore the significant contribution of angiopoietin-2 in cancer progression, not only by stimulating angiogenesis but also promoting metastasis, and provide a mechanism by which breast cancer cells acquire an enhanced invasive phenotype contributing to metastasis.

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P74-5: DISCOVERY OF CANCER METASTASIS HOMING GENES

Anton Wellstein and Marcel Schmidt

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Breast cancer metastasis initiates with cancer cell evasion from the primary site in the breast tissue (i.e., the site of tumor initiation) and progresses with seeding into local lymph nodes and ultimately a distant organ site. It is thought that endothelial surface molecules on the vascular bed of metastatic target tissues contribute to organ specific, metastatic spread of cancer in general and of breast cancer in particular. It is thus hoped to understand the metastatic process at the molecular level and to be able to target molecules critical for this ultimately outcome defining phenotype of breast cancer, i.e., organ metastasis.

To identify genes expressed in human breast cancers that support homing of the tumor cells to distant organs we used established human breast cancer cells with a high propensity to metastasize. We harvested mRNA from human metastatic breast cancer cells and expressed cDNA fragments from this mRNA pool as fusion proteins with gene10 on the surface of phage. We assured that the cDNA sizes were at least 300 nucleotides in length libraries created from this. By this we hoped to cover a significant portion or domains of a given candidate protein that modulates metastasis. Phage expressing these representative libraries were then injected into the circulation of mice and phage particles bound to the endothelia of the lungs, the bone marrow or liver vasculature were rescued thereafter. Over several rounds of selection, the populations rescued were purified further. Blockade of tumor/endothelial attachment by the selected metastasis homing proteins was used as one approach to assess the tumor cell/endothelial interaction. In addition, mRNA expression of the candidate genes in human breast cancer, other cancers (as controls) and normal tissue samples were monitored using in situ hybridization. For this, formalin-fixed, paraffin-embedded tissues were used.

We identified some genes with a known signature for endothelial attachment and some that are differentially expressed in metastatic cancers. We also found genes that were differentially expressed in cancers of different primary origin (breast versus prostate versus colorectal cancer) and differentially expressed in metastatic versus non-metastatic disease and normal tissues. Functional studies in cell culture showed that phage expressing the homing genes can block endothelial attachment of tumor cells.

Conclusions: Primary breast cancers that have a high propensity to metastasize will reveal that phenotype by expressing defined genes that support their homing to distant organ sites. Evaluating such homing proteins as targets to eradicate occult tumor metastases is the next logical step from these results.

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P73-6: MECHANISMS OF MALIGNANT PROGRESS

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The formation of metastatic growths depends on the successful completion of a large number of biological steps that together are termed the invasion-metastasis cascade. We have undertaken to determine what some of the rate-limiting determinants of this process are. Our research has led us to suggest that some of the following are key determinants of the metastatic process occurring at the end of multistep tumor progression. 1. The differentiation program of the normal cell of origin. 2. The nature of the somatic mutations incurred during the course of tumor progression. 3. The nature of embryonic EMT-encoding transcription factors activated during tumor progression. 4. The nature of paracrine heterotypic signals that carcinoma cells receive from the adjacent activated stroma. 5. The ability of primary tumors to send endocrine signals that perturb the bone marrow, enabling the activation and mobilization into the circulation of stromal precursor cells. 6. The ability of cancer cells to recruit stromal precursor cells from the circulation into the tumor-associated stroma, including the stroma of small nests of disseminated cells.

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P73-7: CYCLOOXYGENASE-2 MEDIATED UPREGULATION OF VEGF-C IN HIGHLY METASTATIC BREAST CANCER CELLS DEPENDS ON RECRUITMENT OF SP-1 TRANSCRIPTION FACTOR TO THE VEGF-C PROMOTER SITE

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Background and Objectives: Vascular endothelial growth factor (VEGF)-C is the major lymphangiogenic factor, an overexpression of which has been associated with a

poor prognosis and lymph node metastasis in breast cancer patients. We recently discovered that VEGF-C expression and production is upregulated by endogenous PGE₂ in cyclooxygenase (COX)-2 expressing, highly metastatic human breast cancer cells. Furthermore, endogenous VEGF-C was shown to promote metastasis by stimulation of lymphangiogenesis as well as a direct stimulation of breast cancer cell migration. Since, the promoter for VEGF-C has specific binding sites for each of transcription factors NF-κB, Sp-1, and AP-2, we evaluated whether endogenous expression of COX-2 or exogenously added PGE₂ in highly metastatic MDA-MB-231 breast cancer cells causes recruitment of these transcription factors to the VEGF-C promoter.

Methods: Chromatin immunoprecipitation (ChIP) assay was used under various treatment conditions: (a) asynchronous cells that had been grown in serum-containing medium, (b) serum-starved cells, and (c) serum-starved cells treated with PGE₂. The second set of cells received similar treatments but the cells had been transfected with COX-2 siRNA to knockdown COX-2, and thereby deplete endogenous PGE₂. NF-κB, Sp-1, or AP-2 antibodies were used to immunoprecipitate these proteins for an analysis of their presence on the VEGF-C promoter. A comparative abundance of the transcription factor-bound promoter in the respective immunoprecipitates revealed whether recruitment of these transcription factors was affected by treatment conditions.

Results: ChIP results showed that the recruitment of transcription factors AP-2 and NF-κB was not affected by the treatment of cells with PGE₂, while there was an induction in recruitment of Sp-1 to the VEGF-C promoter in asynchronous cells that was lost significantly when cells were incubated in serum-free medium. Treatment of serum-starved cells with PGE₂ reinstated the level of Sp-1 transcription factor on the promoter suggesting that Sp-1 is one of the transcription factors that contributes to expression of VEGF-C on exposure to PGE₂. In the cells transfected with COX-2 siRNA, a similar pattern was observed suggesting that PGE₂ alone can initiate the recruitment of Sp1 to the VEGF-C promoter in COX-2-downregulated cells.

Conclusion: These results suggest Sp1 to be the primary transcription factor responsible for COX-2-mediated (PGE₂ induced) overexpression of VEGF-C in MDA-MB-231 cells. We shall further validate this conclusion with transient transfection of these cells using a luciferase reporter driven by VEGF-C promoter in presence of these transcription factors.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6096; Canadian Breast Cancer Foundation (Ontario Chapter); and Ontario Institute of Cancer Research.

P73-8: CHANGES IN EphA3 ASSOCIATED WITH BREAST CANCER METASTASIS

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Background: Molecular profiling can be used to detect functional differences in metastatic vs. non-metastatic tumor cells.

Methods: Ten matched pairs of primary breast tumors (PT) and sentinel node metastases (SLM) were harvested and snap-frozen within 15 minutes of resection. Tumor tissue was grossly microdissected from normal tissue. Samples were hybridized to the Affymetrix HU133 2.0 Plus array. Expression profiles from the matched pairs were compared to determine differences for each individual patient, then differences were compared within the total group to determine candidate genes significantly changed within metastatic cells (using Affymetrix Microarray suite software). EphA3 was identified as a gene of interest so immunohistochemistry was used to measure protein expression of EphA3 in matched primary tumor samples and metastatic foci from 41 additional patients with MBC.

Results: EphA3 demonstrated increased gene expression in the lymph node metastasis for 8/10 paired samples. Most metastasis demonstrated loss of protein expression compared to primary tumors (see table).

Conclusion: Metastatic cells have lower levels of EphA3 protein expression compared to primary tumors, and that this loss is not related to changes in transcription.

EphA3 Intensity in metastasis	EphA3 intensity in primary tumors				Total
	0	1	2	3	
0	2	7	9	2	20
1	0	1	6	3	10
2	0	0	8	0	8
3	0	0	0	3	3
Total	2	8	23	8	41
P value	0.003				

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P73-9: NITRIC OXIDE MEDIATES LYMPHANGIOGENESIS AND LYMPHATIC METASTASIS

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Background: Lymphatic metastasis is a critical determinant of prognosis in cancer patients. However, our mechanistic understanding of lymphatic metastasis is still in its infancy. Although correlative clinical studies are present (*Nature Reviews Cancer* 6:521-34, 2006), there are no reports to date on the direct and causal role of nitric oxide (NO) in lymph-angiogenesis and lymphatic metastasis. To this end, we first assessed the ability of exogenous NO to stimulate the growth of lymphatic endothelial cells.

Methods and Results: NO donors (DETA NONOate and Glyco-SNAP-2) induced proliferation of cultured lymphatic endothelial cells in a dose-dependent manner. We next assessed the effect of NO synthase (NOS) inhibition on lymph-angiogenesis using intravital lymphangiography in a recently developed dermal regeneration model (*American Journal of Physiology Heart Circ Physiol* 291:H1402-10, 2006). A NOS inhibitor L-NMMA inhibited regeneration of lymphatic vessels in a 2-mm-wide collagen implant in the mouse tail. Finally, using intravital microscopy to visualize lymph-angiogenesis and the steps of lymphatic metastasis in a newly developed animal model (*Cancer Research* 66:8065-75, 2006), we found that NOS blockade attenuates peritumor lymphatic hyperplasia of vascular endothelial cell growth factor-C-overexpressing fibrosarcomas and decreases the delivery of these metastatic tumor cells to the draining lymph nodes.

Conclusion: These findings indicate that NO plays a causal role in lymph-angiogenesis and, consequently, in lymphatic metastasis. Our findings uncover the underlying biology behind the correlation between NOS and lymphatic metastasis in a number of clinical settings and open the door for potential therapies exploiting NO signaling to treat diseases of the lymphatic system.

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P73-10: THE ROLE OF HOXB7, A MASTER TRANSCRIPTIONAL FACTOR, IN BREAST CANCER METASTASIS

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Metastasis is a multistep process whereby cancer cells gain motility, invade surrounding tissue, adhere to and penetrate endothelial cells of blood vessels or lymphatics to access circulation, and finally, extravasate at distant organs. Metastasis is the major cause of morbidity and mortality associated with breast cancer. The inability to effectively predict, prevent, and treat metastatic breast cancer is a major problem in breast cancer research. Preliminary studies from our laboratory show that HOXB7, a transcription factor, is overexpressed in both primary breast tumors and in bone metastases. The goal of these studies is to investigate whether the overexpression of HOXB7 confers invasive and metastatic capability to breast tumor cells and to study the underlying mechanism with the long-term goal of developing novel and effective therapies. To study the function of HOXB7 in vivo, we generated MMTV-Hoxb7 transgenic mice. Although a subtle abnormal mammary gland phenotype was observed, single transgenic Hoxb7 mice did not develop mammary tumors. To determine if Hoxb7 plays a role in tumor promotion rather than in tumor initiation, we crossed the Hoxb7 transgenic mice with MMTV-Her2/neu transgenic mice. In mice carrying both transgenes, we found that Hoxb7 played a dual role in neu-induced mammary tumorigenesis. Contrary to prediction, compared to the single Her2/neu-overexpressing transgenic mice, in double transgenic mice overexpression of Hoxb7 along with Her2/neu delayed tumor onset and lowered tumor multiplicity. However, once the tumors appeared, their growth was faster and they metastasized to the lung at a higher frequency. Our data demonstrates, for the first time, that deregulated expression of Hoxb7 in mammary tumor cells can significantly modulate neu-oncogene induced tumorigenesis in vivo. Hoxb7 may act as an antiproliferative gene in the early stages of carcinogenesis, but as a potent pro-metastatic gene in the later stages of tumor progression. Our previous data supported the upregulation of multiple growth factor receptor pathways, such as estrogen receptor and epidermal growth factor receptor in HOXB7-overexpressing breast cancer cells. We are currently investigating antagonists of these pathways in multiple cell lines established from primary mouse tumors arising in Her2/neu and Her2/neu x Hoxb7 mice. Furthermore, to determine the influence of high HOXB7 expression in human breast cancers, we performed in silico analysis of published microarray data. The results showed that high levels of HOXB7 predicted a poor outcome in HER2-positive ($p=0.046$), but not in HER2-negative breast cancers ($p=0.94$), lending support to the relevance of our findings in the dual transgenic mouse model.

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P73-11: BREAST CARCINOMA CELLS IN THE METASTATIC ENVIRONMENT RE-EXPRESS E-CADHERIN AS A SURVIVAL MECHANISM

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Epithelial cadherin's (E-cadherin) transcriptional silencing in most advanced tumors, due to promoter methylation, enables tumor cells to disseminate from the primary mass. However, E-cadherin-positive metastatic carcinoma foci do originate from mainly E-cadherin-negative primaries. It is unknown if this is due to dissemination of a minor population of E-cadherin positive cells or re-expression of E-cadherin during metastasis. Here, we demonstrate that co-culture of invasive E-cadherin-negative breast cancer cells with hepatocytes, the second most common site of soft tissue metastasis for breast cancer, triggers an epigenetic reversion in breast cancer cells resulting in demethylation of the E-cadherin promoter and subsequent expression on the protein level. Demethylation of the E-cadherin promoter is coupled to proliferation of the cancer cells and is not the result of a global demethylation program. We show a similar time-course for E-cadherin upregulation in 3 of 11 of primary human breast cancer explants co-cultured with primary hepatocytes. Further, we show that E-cadherin ligation between breast cancer cells and hepatocytes is functional and activates the canonical MAPK and Akt pathways in these cancer cells. This E-cadherin binding makes the breast cancer cells more resistant to apoptotic signals and chemotherapies. Our epigenetic-reversion hypothesis for E-cadherin represents not only a paradigm shift in the current thinking that absence of E-cadherin is fundamental for metastasis, but would also reveal mechanisms underlying the failure to readily treat the early stages of metastatic disease in breast cancer patients with existing therapies.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0403.

P73-12: ROLE OF β -CATENIN SIGNALING IN BREAST CANCER INVASIVENESS AND METASTASIS

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The major determinant of breast cancer mortality is the development of secondary tumors or metastases. The mechanisms by which primary tumors become invasive are poorly understood. Recently, epithelial-mesenchymal transition (EMT) model has been proposed to play a major role in metastasis. In this study, we examined the role of β -catenin signaling pathway in the EMT process. The major β -catenin binding partner is the epithelial cell adhesion molecule E-cadherin that is often downregulated during carcinoma progression. In addition, loss of E-cadherin is highly associated with the metastatic spread of tumors. However, the precise mechanism and molecular basis of metastasis promotion by E-cadherin loss and its impact on β -catenin activity is less clear. To be able to address this question, we utilized RNA interference (RNAi) against E-cadherin in a human mammary epithelial cell line. Loss of E-cadherin induced the EMT with concomitant activation of β -catenin, and conferred upon these cells the ability to metastasize. To assess the functional importance of β -catenin activation, we inhibited β -catenin expression using RNAi. Upon β -catenin suppression, levels of EMT-induced genes diminished, suggesting a necessary role for β -catenin in their induction. To assess the role of β -catenin in an in vivo model, we performed tail-vein injections with β -catenin suppressed cells and observed that metastasis was greatly reduced. Additionally, in vitro assays showed that β -catenin is necessary for the acquisition of invasiveness and anoikis resistance by the cancer cells. Our findings suggest that loss of E-cadherin in human tumors may have wide-ranging transcriptional and functional consequences that are mediated by at least in part by the β -catenin protein.

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P73-13: THE INFLUENCE OF NEURONAL ACTIVITY ON BREAST TUMOR METASTASIS TO THE BRAIN

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Fatalities in patients with breast cancer are often a result of metastasis of the breast tumor to the brain. The growth and dissemination of metastases are critically dependent on the structure of the extracellular matrix (ECM) of the target organ, and many clinical approaches to metastasis treatment and prevention have targeted proteinases that degrade this matrix. It has recently been shown that the motility of synaptic structures in the brain is modulated by neuronal activity through a pathway that involves the serine proteinase tissue plasminogen activator (tPA) and results in the ultimate degradation of the ECM. We have been using the insights gained from our studies of synaptic motility, as well as the abundance of clinically approved agents that alter neuronal activity, to investigate a novel therapeutic avenue for inhibition of breast tumor brain metastases.

We are currently studying the ability of a human-derived breast tumor cell line that has been optimized for the ability to metastasize to the brain (MBA-MB-231-BR) to cross the blood brain barrier and grow within the brain under different treatment conditions. We are testing the ability of clinically approved stimulants that are used to treat neurological disorders to reduce the dissemination and growth of this cell line in immunocompromised mice. Additionally, we are exploring the involvement of the tPA-plasmin axis in the metastatic-hindering effects of these drugs and characterizing changes in the diffusive hinderance and ECM composition following treatment.

We hypothesize that normal mechanisms used by neurons to alter the ECM as a result of changes in neuronal firing also affect breast tumor metastatic extravasation, growth, and dissemination. This innovative insight allows us to immediately test the antimetastatic ability of three drugs already approved for use in the clinic and hence offers the possibility of extremely rapid clinical application of our ideas. Beyond the short-term benefit derived from testing clinically approved drugs for their antimetastatic potential, this work will establish an absolutely novel and fundamentally new paradigm for metastatic control whereby interventions that modify neuronal activity offer promising candidates as interventions that modify tumor growth and invasion. This will lead to the investigation of other reagents and interventions not yet explored in this work. Last, the efforts of this work to explore the fundamental mechanisms underlying activity-dependent ECM modification and its metastatic effects represent an entirely new field of study and may produce novel therapeutic targets for treatment of breast tumor metastases to the brain.

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P73-14: CD44 AND METASTATIC POTENTIAL IN BREAST CANCER CELLS

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Metastases and drug resistance are the major causes of treatment failure in breast cancer. CD44 is a transmembrane receptor implicated in increasing drug resistance and metastatic potential of breast cancer cells. Matrix metalloproteinases (MMPs) are a group of endopeptidases that degrade most of the extracellular matrix (ECM). MMPs play an important role in invasion and metastasis of tumors, in which they are frequently overexpressed. Because some MMPs are known to bind CD44 (e.g., MT1-MMP and MMP-9) or are regulated by the CD44-ligand interaction (e.g., MMP-2 and MMP-9), we investigated the effect of CD44 expression on MMPs expression and activity in CD44-negative breast (MCF-7) cancer cell lines. CD44 expression levels were confirmed by RT-PCR and immunoblotting. The in vitro migration and invasion of the clones was determined. The activity of MMPs was assayed by gelatin and fibrinogen zymography. MCF-7/CD44s showed a significant increase in matrigel invasion, multidrug resistance and up-regulation of MMP-9 and MMP-3 compared to MCF-7. No MMP-2 activity was detected for either MCF-7 or MCF-7/CD44s clone. MT1-MMP expression was also up-regulated as shown by RT-PCR. Because CD44 can be enzymatically processed into multiple peptide fragments and its cytoplasmic tail has been detected in the nucleus, we examined whether the presence of the CD44 cytoplasmic tail influences the up-regulation of MMPs. For this purpose, the cell lines MCF-7 [CD44 (-), P-gp (-)]; MCF-7/Adr [(CD44 (+), P-gp (+)); MCF-7/CD44s (a CD44s stable transfectant clone that also induces P-gp expression) and BC19 (an MDR1 stable transfectant clone, that expresses P-glycoprotein and not CD44) were transiently co-transfected with a pcDNA3.1/CD44s construct in which the cytoplasmic tail was absent (CD44-tailless) or with a construct carrying the entire CD44s sequence (CD44-wt), or just the intracytoplasmic tail (CD44-ICD) in a CAT reporter construct driven by the MMP-9 promoter sequence. Reverse-transcription (RT)-PCR analysis of these transfectants revealed that in MDR1 gene-expressing cell lines, the expression of CD44-tailless decreased the expression of CAT. This effect was not observed when vector plasmid alone (pcDNA3.1) was expressed in these cells. The expression of CD44 wt increased CAT expression. The up-regulation of the MMP-9 promoter driven CAT expression was independent of the expression of EMMPRIN, a transcriptional regulator of MMPs expression. These results suggest that CD44 upregulates the expression of MMPs in breast cancer cells through its intracytoplasmic tail and this regulation is EMMPRIN independent.

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P73-15: TRANSFORMING GROWTH FACTOR β PATHWAY ANTAGONISTS INHIBIT HUMAN BASAL CELL-LIKE BREAST CANCER METASTASES TO LUNG AND BONE

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Transforming Growth Factor β (TGF β) is believed to promote tumor progression by enhancing invasion, metastasis, and angiogenesis, and by suppressing anti-tumor

immunity. To investigate the possible clinical utility of TGF β antagonists in metastatic breast cancer, we examined the effects of two types of antagonists of the TGF β pathway (1D-11, a mouse monoclonal pan-TGF β neutralizing antibody and LY2109761, a chemical inhibitor of TGF β type I and II receptor kinases) on experimental metastases of basal cell-like breast cancer in vivo. For this purpose, we used sublines of basal cell-like MDA-MB-231 human breast carcinoma cells that preferentially metastasize to lungs (MDA-231-4175TR) or bone (MDA-231-SCP2TR and 2860TR). Both antagonists were able to block TGF β -induced phosphorylation of the receptor-associated Smads, Smad 2, and -3, in each of these cell lines. In addition, treatment with LY2109761 but not with 1D11 induced dephosphorylation of activated Smads. TGF β failed to inhibit anchorage-dependent growth of any of these cell lines. However, TGF β stimulated in vitro migration of MDA-231-SCP2TR, 2860TR, and MDA-231-4175TR cells by 1.96-, 1.42-, and 1.66-fold, respectively ($p < 0.05$). In addition, TGF β stimulated invasiveness into Matrigel[®] of MDA-231-SCP2TR, 2860TR, and MDA-231-4175TR by 1.94-, 1.39-, and 1.52-fold, respectively ($p < 0.05$). This in vitro stimulation of migration and invasion by TGF β was inhibited by both antagonists, indicating that these processes are partly driven by TGF β . Following left intracardiac injection in female athymic nude mice, MDA-231-SCP2TR and 2860TR cells metastasize to bones. Conversely, MDA-231-4175TR injected into the tail vein metastasize to lungs. Moreover, all three cell lines express a luciferase reporter gene, which allows us to monitor the process of metastasis using in vivo bioluminescence imaging. Beginning 1 day following tumor cell injection, mice were treated with 5 mg/kg 1D-11 given intraperitoneally three times per week for 7 weeks. Treatment with 1D-11 antibody significantly reduced the burden of MDA-231-SCP2TR or 2860TR-derived metastases to bones as well as MDA-231-4175TR-derived metastases to lungs by approximately 40%. In separate experiments, mice inoculated with MDA-231-4175TR or with SCP2TR cells were treated with 50 mg/kg LY2109761 twice daily by gavage for 5 weeks. LY2109761 treatment also significantly reduced the burden of MDA-231-4175TR-derived lung- and MDA-231-SCP2TR-derived bone metastases by approximately 40%. In aggregate, these results support the notion that TGF β plays a role in both bone- and lung metastases of basal-like breast cancer and that inhibiting TGF β signaling results in a therapeutic effect independently of the tissue-tropism of the metastatic cells. Targeting the TGF β pathway holds promise as a novel therapeutic approach for metastatic basal-like breast cancer.

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P73-16: ENHANCED METASTATIC POTENTIAL OF KERATINOCYTE GROWTH FACTOR-TRANSFECTED HUMAN BREAST CANCER CELLS IN A MOUSE XENOGRAFT MODEL

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Background: We previously reported that keratinocyte growth factor (KGF) rapidly enhances the motility of estrogen receptor (ER)-positive breast cancer cells (Zang, XP and Pento, JT. *Clin. & Exptl. Metastasis* 18: 573-80, 2001). Others have demonstrated that KGF treatment in rodent species produced mammary ductal hyperplasia and eventually metastatic cancer development. Further, we have shown that the KGF-induced motility effect is mediated by the KGF receptor (KGFR) via receptor tyrosine kinase (TK)-mediated Erk1,2 signal transduction pathway (Zang, XP, et al. *Clin. & Exptl. Metastasis* 21: 437-43, 2004). Based on these observations, we hypothesize that KGF/KGFR enhancement is an early signal in breast cancer metastatic progression. The present study developed a mouse xenograft model to examine the influence of KGF on the growth and metastasis of breast cancer cells.

Material and Methods: MCF-7 cells, containing a GFP reporter, were transfected with a KGF-cDNA vector and a stable clone (T8) producing elevated levels of KGF, and GFP was selected for use in this study. MCF-7 cells containing only the GFP reporter were used as a KGF-negative empty vector clone (EV) in this study. Western blotting, ELISA, and fluorescence microscopy were used to confirm the expression of KGF and/or GFP in the transfected cells. In the xenograft experiments, 5×10^6 viable T8 or EV cells were mixed with 0.2 mL Matrigel and injected s.c. into the mammary fat pads of female nu/nu balb/C mice at 6 weeks of age to create an orthotopic-type xenograft model. An estradiol pellet was implanted into each animal 48 hours prior to cancer cell implantation.

Results: The T8 clone used in this study was shown to be stably transfected and to express the GFP reporter and nM quantities of KGF approximately 500–1,000-fold greater than the EV control cells. An examination of these T8 cells demonstrated that the proliferation and motility of the KGF/GFP-transfected T8 clone was 2-fold greater than either the WT or EV cells. Also, the morphology of the T8 clone revealed more pseudopodia, membrane extensions, and cell scattering than either the WT or EV cells. At 80 days following cancer cell implantation, the tumor xenografts in mice implanted with the T8 cells were 4-fold larger than xenografts in the control animals implanted with the EV cells. Tumors were measured by both external caliper and a whole-animal fluorescence imaging system. Fluorescence micrographs of liver and lung sections revealed numerous micro-metastases in tissue sections from mice with T8 implanted cells while very few micro-metastases were found in the liver and lung tissue from control mice.

Discussion: Wild-type MCF-7 cells are known to be poorly metastatic in mouse xenografts. Thus, these results indicate that an elevated level of KGF in the tumor microenvironment enhances cancer cell proliferation, tumor growth, and the development of micro-metastases. The results support the concept that KGF may be an important early mediator of tumor growth and metastasis. The xenograft model developed in this study should permit the accurate assessment of antimetastatic activity of novel compounds that may be used to prevent or impede KGF-mediated breast cancer growth and metastasis.

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P73-17: UNDERSTANDING STROMAL-EPITHELIAL INTERACTIONS OF BRMS1-SUPPRESSED METASTATIC BREAST CANCER CELLS AT THE SECONDARY SITE USING THREE-DIMENSIONAL TISSUE CULTURE

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BRMS1 prevents metastasis of breast cancer cells to lung and bone without suppressing tumorigenicity. Data indicate that cells arrive in the lung, but do not grow into macroscopic lesions. That cells grow in the orthotopic site, but are suppressed at secondary sites indicates that local microenvironment controls suppression of metastasis. We

hypothesize that suppression by BRMS1 is due to microenvironmental-specific changes in cellular communication or signaling. Three dimensional (3D) culture recapitulating lung and mammary gland microenvironments will be used to determine morphological and signaling mechanisms resulting in BRMS1-induced suppression of metastasis. MDA-MB-231 cells with and without overexpression of BRMS1 have been grown in 3D mono-cultures. The BRMS1 expressing cells have spheroid morphology reminiscent of normal mammary epithelial cells compared to the parental 231 cells that appear as disorganized, aggressive structures. Proliferation, apoptosis, polarization of epithelial cells and epithelial to mesenchymal transition are currently being examined and will be presented. Concurrently, 3D co-cultures with fibroblasts isolated from lung and mammary gland are being developed to understand why BRMS1 expressing cells are dormant in the lung, but not the mammary gland. Proliferation, apoptosis, polarization of epithelial cells and epithelial to mesenchymal transition will be examined and presented. Previous studies have identified two possible mechanisms of BRMS1 metastasis suppression. BRMS1 expression increases gap junctional intercellular communication and decreases phosphoinositide 3,4 bisphosphate and EGFR expression. Co-cultures will be examined for expression and phosphorylation of EGFR, PDGFR, AKT, and ERK. Cell/cell communication will be examined by connexin 32/43 staining and dye transfer through gap junctions. These studies will facilitate understanding of the interaction of metastasis-suppressed breast cancer cells with the microenvironment at primary and secondary sites. These data may allow design of targeted therapy to prevent lung metastasis, thereby improving quality of life and survival rates of breast cancer patients.

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